Negative Regulators of Chromosome Replication in the Dimorphic Bacterium *Caulobacter crescentus*

William John Spencer

Department of Microbiology and Immunology,

McGill University, Montreal, Quebec

December, 2006

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Your file Votre référence
ISBN: 978-0-494-32387-8
Our file Notre référence
ISBN: 978-0-494-32387-8

ABSTRACT

Ph.D. William Spencer Microbiology and Immunology

Caulobacter crescentus provides an accessible system for investigating the regulation of chromosome replication and cellular development. The *Caulobacter* cell cycle produces a free-swimming swarmer cell and a sessile stalk cell. In swarmer cells, chromosome replication is selectively repressed while stalk cells are committed to chromosome replication.

In *Caulobacter,* chromosome replication is repressed, in part, by the binding of the response regulator CtrA to five binding sites (**a-e**) within the *Caulobacter* origin of replication (*Cori*). Periodic phosphorylation of CtrA stimulates binding to the consensus sequence TTAA-N7-TTAA (N= any nucleotide) found in *Cori* and many cell-cycle regulated genes. This thesis presents an alternate mode of CtrA binding, namely, that phosphorylation does not stimulate binding to a specific class of CtrA-regulated promoters. This work shows that CtrA and CtrA-phosphate bind to two *ctrA* promoters with equal and weak affinity. As well, *in vivo* binding assays reveal that a non-proteolyzable CtrA allele (CtrAΔ3) can occupy the *ctrA* promoters continuously without altering the temporal regulation of these promoters. The data suggest phosphorylation, while not increasing affinity for weak CtrA binding sites, provides allosteric signals that permit the recruitment of components required for transcription.

The proposed allosteric mechanism of CtrA-regulated transcription may also be important for CtrA-mediated repression of chromosome replication.

Chromatin Immunoprecipitation assays (ChIP) allow for the sensitive detection of specific protein/DNA complexes *in vivo.* ChIP reveals that CtrA binds to *Cori* in swarmers but not in stalk cells when chromosome replication commences. The protein chaperone, ClpX, was recruited to *Cori* prior to the start of S-phase and correlates with the loss of CtrA binding to *Cori.* Expression of a non-proteolyzable CtrAΔ3 allele showed increased affinity for *Cori* DNA. The increase in CtrAΔ3 binding stimulated a corresponding increase in ClpX binding to *Cori.* This evidence suggests that ClpX recruitment to *Cori* is likely CtrA-dependant. The absence of CtrA binding in stalk cells suggests other mechanisms may be required to prevent re-replication in stalk cells.

An analysis of the *Caulobacter* genome identifies two DnaA-like genes. The first, *cdl-1*, is a homolog of the *E. coli hda* gene, a protein essential for regulated inactivation of DnaA (RIDA). The second, *cdl-2*, is a novel gene restricted to the alpha-proteobacteria group and whose function is unknown. Overexpression of either gene in *Caulobacter* produced filamentous cells that could not divide. DNA synthesis in these cells is also impaired and suggests the intracellular concentrations of these two proteins are important for coordinating proper cell cycle progression.

RÉSUMÉ

Ph.D. William Spencer Microbiology and Immunology

Caulobacter crescentus procure un système accessible pour étudier la régulation de la réplication de chromosomes et du développement cellulaire. Le cycle cellulaire du *Caulobacter* produit une cellule de swarmer mobile et une cellule de stalk immobile. Dans les cellules de swarmer, la réplication de chromosomes va arrêter sélectivement son développement tandis que les cellules de stalk se consacrent entièrement à la réplication de chromosomes.

Dans le *Caulobacter,* la réplication de chromosomes est réprimée, en partie, par le lien de réponse du régulateur CtrA à cinq liens (**a-e**) a l'intérieur du *Caulobacter* de la réplication d'origine (*Cori*). La phosphorylation périodique de CtrA stimule le consensus du lien TTAA-N7-TTAA (N= tous types de nucléotides) trouvé dans *Cori* et plusieurs gènes régulés du cycle cellulaire. Cette thèse présente un deuxième model de liens du CtrA, c'est a dire, que la phosphorylation ne stimule pas le lien à une classe spécifique des promoters réguler du CtrA. Ce travail démontre que le CtrA et le phosphate CtrA se lient à deux promotors *ctrA* avec une faible et égale ressemblance. De plus, Les expériences *in vivo* de liens révèle que les allèles non dégradables de CtrA (CtrAΔ3) peuvent lier les promotors *ctrA* sans interruption de la régulation temporelle de ces promotors. Les données suggèrent que la phosphorylation, tout en n'augmentant pas la ressemblance pour les faibles sites de liens du CtrA,

peuvent fournir les signaux allostériques pour l'association des composantes qui sont exigées lors de la transcription.

Le mécanisme allostérique proposé de la transcription régulé du CtrA peut également être important pour bloquer la réplication de chromosomes du CtrA. Les expériences d'immunoprecipitation de Chromatine (ChIP) tiennent compte de la détection très précise des complexes spécifiques de protéines d'ADN *in vivo.* Le ChIP indique que le CtrA se lie à *Cori* dans les swarmers mais pas dans les cellules de stalk quand la réplication des chromosomes débute. La protéine qui assiste le ClpX se recrutait à *Cori* avant le début de la phase S et corrobore la perte du CtrA se liant à *Cori.* L'expression de la non dégradation de l'allèle CtrAΔ3 montre des accroissements pour l' ADN de *Cori.* L'augmentation des liens de CtrAΔ3 ont stimulé une augmentation correspondante des liens ClpX à *Cori.* Cette évidence suggère que le recrutement de ClpX à *Cori* est probablement dépendant du CtrA. L'absence des liens CtrA dans les cellules de stalk suggère que d'autres mécanismes puissent être exigés pour prévenir la sur réplication des cellules de stalk.

Une analyse du génome de *Caulobacter* identifie deux gènes de DnaA semblable. Le premier, le *cdl-1*, est un homologue du gène de *E. coli hda.* C'est une protéine essentielle qui empêche l'activité de l'initiateur de protéine DnaA. La seconde, le *cdl-2*, est un nouveau gène restreint au groupe d'alpha proteobacteria et dont le fonctionnement est inconnu. La surabondance de l'un ou l'autre des gènes du *Caulobacter* produit des cellules filamenteuses qui ne peuvent pas se diviser. L'ADN de synthèse dans ces cellules est également altéré

et suggère que les concentrations intracellulaires de ces deux protéines sont importantes pour coordonner la progression appropriée du cycle cellulaire.

CONTRIBUTION OF AUTHORS

All experiments were performed by the author except the DnaseI footprint assay shown in Figure 1 of Chapter 2 which was originally performed by Rania Siam, Ph.D. in 2000 and reconfirmed by our lab technician, Marie-Claude Ouimet, in 2004.

Chapter 2 and Chapter 3 are the following manuscripts pending submission.

Spencer W. J., Siam, R., Ouimet, M.C. & G.T. Marczynski. (2006) Phosphorylation does not recruit response regulator CtrA to a new class of *Caulobacter crescentus* promoters. Manuscript to be submitted.

Spencer W. J. & G.T. Marczynski. (2006) ClpX Chaperone Recruitment to the *Caulobacter crescentus* Replication Origin. Manuscript to be submitted.

ACKNOWLEDGEMENTS

I would like to thank Madani Thiam, Boris Gorbatyuk, Oleh Tanchek, Cezar Khursigara, Michael Blank, Patrick Bastedo, Camille Sayegh, Mark Arbing, James Scrivens, and David Alexander who acted as my moral compass throughout my time at McGill.

To my lab I would like to extend a special thanks to M-C Ouimet, Mr. S.M. Shaheen, Karen Brassinga, and Rania Siam, for their technical support and outstanding friendship.

I would personally like to thank Dr. Herve LeMoual and Dr. Benoit Cousineau, members of my Ph.D. advisory committee including the members of my comprehensive exam committee, Dr. James Coulton, Dr. Chantale Autexier, and Dr. Bernard Turcotte.

To the world wide web of *Caulobacter* researchers I would like to especially thank Dr. James Gober. Dr. Urs Jenal, Dr. Christine Jacobs-Wagner, Dr. Dicken Alley, Dr. Yves Brun and finally to Dr. Craig Stephens at UC Santa Clara, you were all a source of inspiration. To those in the *Caulobacter* trenches I want to recognize Arun Divakaruni, Jennifer England, Rachel Muir, Sherry Wang, Aaron Hinz, David Larson, Christopher Smith, Thomas Fuchs, Martin Ackerman, Ellen

Judd, Mike Laub and Rasmus Jensen, thanks for your perspectives and your protocols!

To my Masters supervisor Dr. John Hiscott for convincing me to come to McGill so many years ago and to Dr. Robert Murgita for encouraging my decision to join the Ph.D. program in 2001.

To the Lyman Duff Crew Past and Present; Adelaida Gomez, David Gregory, Alicja Cieslak, Mark Arbing, Rachel Morse, Juliene DeFlasseux, Kyrillos Ragzm, Gloria Lei, Sarah Sanowar, Kamilla Belhocine, Valerie LeSage, Mirna Nascimento, Deb Stewart, Fransisco Ferriera, Kirsty Salmon, Ian Siboo, and Julie Guzzo.

This thesis would not have been possible without the support of my wife, Anouk Adam, my two children, Thomas and Sarah, my parents William and Rita, including my brother Jason, who provided unending encouragement during the non-linear pursuit of my degree(s).

Finally to my supervisor Greg Marczynski, your generosity was second only to your extraordinary intelligence, thank you for your patience and guidance over the years.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF TABLES

CHAPTER 2

CHAPTER 4

CHAPTER I: Review of the Literature

Introduction

The original replicon model of Jacob, Brenner, and Cuzin proposed that replication is controlled from a specific location, the "replicator", and that it is recognized by a specific positive regulatory protein, the "initiator" (1). In current terms, the model suggests that specific DNA sequences permit the entry of the replication machinery and that special proteins (in addition to DNA polymerases) are required for regulated DNA replication. While the mechanisms of DNA replication are still incomplete, the growing list of replication proteins characterized continues to support the essential replicon model in both eukaryotes and prokaryotes. Of growing interest is a family of ATPases, found in all three kingdoms of biology, that play an intimate role in controlling the events of DNA replication (2). This family of ATPases is designated AAA+, or ATPases Associated with various cellular Activities, an important subject not only in this thesis but for future research in the replication field. The original replicon model also stressed the importance of positive acting replication proteins that promote DNA replication. We now know that negative acting proteins are also important to prevent extra replication, and this is a major theme of this thesis.

This literature review is broken into four major parts. The first part presents the major advancements in our understanding of DNA replication in the best studied systems of bacteria and yeast and it compares and contrast their mechanisms. The second part focuses the negative mechanisms that restrict chromosome replication to once per cell-cycle. This part reveals that regulation of *E. coli* replication occurs through a negative feedback mechanism on the initiator (DnaA). In contrast to *E. coli,* yeast temporally

segregates the licensing of replication and the initiation event that uses the license. This contrast reveals a major regulatory dichotomy between prokaryotes and eukaryotes. The third part introduces chaperones and how they function to control DNA replication in a variety of systems including phages and plasmids. This part also draws inferences on how chaperones might play a role in bacterial chromosome replication. The review ends with an analysis of *Caulobacter crescentus* to help us uncover the fundamental nature of this organism to answer important questions underlying the natural history of DNA replication initiation and its role within the larger context of cell cycle regulation. This review prepares for the remaining chapters where I present experiments designed to increase our understanding of negative regulators of chromosome replication in the dimorphic bacterium *Caulobacter crescentus*.

I. Current Models of Chromosome Replication

1.1 E. coli Replication

For *E. coli* and most prokaryotic organisms studied, replication initiation occurs at a unique location within a single circular chromosome. In *E. coli,* this region is referred to as *oriC* and disruptions of this region can typically be rescued by the integration of a replicating phage or plasmid origin. The cloned *E. coli* origin also supports autonomous replication of plasmids lacking functional origins. This property of the origin indicates that the minimal origin sequence contains all the elements necessary to permit replication. It should also be noted that DNA replication and cell viability are closely linked because cells that fail to replicate, even though the genes responsible for growth and development are intact, quickly lose viability. Although the detailed reasons for this are not known, the loss of viability suggests that faithful completion of chromosome replication is an important checkpoint for cell cycle progression.

The minimal sequence requirement for *oriC* is a 245 bp DNA fragment (3) capable of supporting autonomous plasmid replication. *oriC* contains a number of important elements for the binding of replication proteins (Figure 1) that are conserved among the enteric bacteria (4). The most conspicuous elements are the five binding sites (R1-R4, M) for the initiator protein DnaA (5), arranged as 9-mers having the consensus TTATNCACA (discussed below). The leftward region of *oriC* has three 13-mer direct repeats (L, M, and R) that are rich in AT residues (6). During the initiation of replication, DnaA contacts the 13-mers and separates the DNA strands first at R followed by M and L

(7). Additional conspicuous elements of *oriC* are the GATC sequences which are targets for Dam (DNA adenine methyltransferase). Methylation of these Dam sites is important for restricting the timing of replication. *E. coli dam*$^-$ strains are viable, however unlike WT cells, *dam*$^-$ cells have asynchronous chromosome replication (8,9). It has been demonstrated that hemimethylated origins (where only the parental strands are methylated) are sequestered to the membrane and provide an important temporal mechanism for replication (10,11). *oriC* also contains binding sites for the histone-like proteins that play an important role in the assembly of the pre-replication complex (12). Three proteins have been characterized at *oriC*, namely Hu and IHF which promote assembly of the pre-replication complex (13,14) and Fis, an antagonist of DNA replication (15). The primary function of these proteins may be through the introduction of specific bends in the DNA helix that promote communication between distal sites within *oriC*. While histone-like proteins are not essential when mutated individually, double mutants do not support autonomous plasmid replication (16). The minimal *oriC* is also flanked by two promoters, *mioC* and *gidA,* and transcription from these promoters is a necessary component in stimulating replication. The proposed explanation is that RNA polymerase, as it unwinds the template DNA for transcription, also provides important topological changes in the vicinity of *oriC* which presumably assist DnaA in melting this region (17).

1.1.1 From Initiation to Elongation

The mechanism of replication initiation has largely been uncovered by *in vitro* studies with *oriC* plasmids in reconstituted replication reactions using purified proteins (17).

The initial stages of DNA replication require three members of the AAA+ family, namely DnaA, DnaB, and DnaC (Figure 2). The primary step in replication is the binding of DnaA to the origin. DnaA is an essential gene in most organisms studied. However, under certain genetic conditions loss of the initiator function (DnaA – see below) is tolerated in the presence of specific RNase H suppressor mutation (18,19) and replication initiation is permitted. In this instance, deficiencies in RNaseH lead to single stranded DNA at sites of mRNA transcription which act as surrogate targets for the DNA replication apparatus. However, under these conditions, DNA replication is no longer cell cycle regulated and cell growth is dramatically impaired, further proof that proper coordination of DNA replication is an essential function in growing cells. The stoichiometry of DnaA in the pre-priming complex is approximately 10 molecules of DnaA (20). During the melting process this number increases to approximately 20 (5). The form of DnaA is also important. DnaA is found in both DnaA-ATP and DnaA-ADP forms and only the ATP-bound form of DnaA is able to promote replication initiation (21,22). While DnaA-ADP recognizes all five binding sites within *oriC,* only the binding of DnaA-ATP leads to productive melting of the AT-rich region (23). Direct evidence for DnaA binding to the E. coli *oriC* has been demonstrated by both filter binding assays (24) and DNase I footprint assays (23). Efficient unwinding of the duplex by DnaA-ATP also requires negative supercoiled DNA and the activities of the histone-like proteins HU or IHF (23,25).

The emergence of single stranded DNA during origin unwinding promotes the transfer of the helicase DnaB by DnaC (21,26). However, DnaB has relatively low affinity for DNA

and requires the activity of both DnaC, as a transporter for loading, and DnaA for stabilizing DnaB at the origin (26-28). DnaB and DnaC both form hexameric ring structures, a typical feature of AAA+ members, and this structure is essential for the catalytic activity of the individual subunits. Like many processes in DNA replication, DnaC activity requires ATP (27,29-31) and its ATPase activity is required for the release of DnaB (27,32). The ratio of DnaC to DnaB is also important, since excess DnaC exerts a toxic effect by inhibiting DnaB activity (32). *oriC* recruits two DnaB hexamers and the additional recruitment of DnaA during the pre-priming step may restrict the loading of extra DnaB molecules (20). Once loaded, DnaB catalyzes further unwinding of the duplex and permits the loading of single stranded binding proteins (SSB) which maintains the melted state of the DNA (33). The ATP-dependent topoisomerase, DNA gyrase, is also recruited to alleviate positive supercoils, a form of DNA tension, that blocks forward movement of the replication forks (34). Since replication fork movement is bidirectional both *in vivo* and *in vitro*, two DnaB complexes are required (33,35) Upon uwinding of *oriC* by DnaB, the primase complex DnaG is loaded and RNA primers are synthesized, providing a template for DNA Pol III holoenzyme elongation (33). It was alluded to earlier that negative super-coiled DNA is a requirement for *E. coli* replication. It is clear that chromosome replication is dependent on transcription by RNA polymerase and can directly assist DnaA in *oriC* unwinding (18). RNA polymerase also affects replication by altering template structure *in vitro* (25,34) and RNA polymerase may act similarly *in vivo* (36-38).

1.1.2 Elongation and DNA Synthesis

The cross-over point from initiation to elongation is primarily mediated by the activity of the DnaB helicase as it coordinates the assembly of the other replication fork components (Figure 3). DnaB belongs to a family helicases that are involved in DNA replication, recombination, and repair. There are at least 14 helicases in the *E. coli* genome, each dedicated to a specialized function (39). However, DnaB is the major helicase responsible for chromosome replication (33,40). The polarity of DnaB movement on DNA (5'-> 3') means that this helicase associates with only one strand, namely the lagging strand (41). DnaB has a unwinding rate of 700bp/sec (42). DnaB is also responsible for recruiting and activating primer synthesis by the DnaG primase. DnaG synthesizes ribonucleotide primers on SSB coated single stranded DNA (43). In general, specific binding sites for DnaG are not required but rather DNA secondary structure stimulates DnaG binding. In addition, ATP binding but not hydrolysis is important for DnaB-dependent activation of DnaG (44). While physical evidence for DnaB/DnaG interactions have not been demonstrated, evidence does suggest DnaB may provide important alterations in the DNA secondary structure required for DnaG recruitment to the lagging strand template (17).

The next step in the transition to elongation is the positioning of the DNA polymerase III holoenzyme. This large, multi-subunit complex is comprised of two major subassemblies, the core polymerase and the accessory factors. As will be described later, these proteins also share many similarities with the eukaryotic replication enzymes. The core polymerase consists of three tightly bound subunits including the alpha subunit

(*dnaE*) which comprises the basic catalytic subunits (45), a 23-kd proofreading exonuclease (*dnaQ*) (46) and the theta subunit (*holE*) whose exact function is not clear but it may enhance epsilon proofreading activity (47). The core polymerase by itself can only synthesize short DNA strands, because it readily dissociates from template DNA. To increase its processivity, accessory proteins are recruited. The gamma and tau proteins are translational frameshift products of the *dnaX* open reading frame (48,49) and help to link DNA Pol III as a dimer (50,51). The gamma subunit also associates with other components, namely, delta, delta', chi, a psi (HolA, B, C, D) to form the clamp loader or "gamma complex." The gamma complex is a DNA-dependent ATPase that loads the beta subunit (DnaN) (52). X-ray crystallographic studies reveal that the beta subunit exists as a dimer, forming a closed ring around the duplex DNA (53). Therefore, the loading activity of the gamma complex would first open then close the beta subunit ring onto duplex DNA. In this way the beta subunit has been referred to as a clamp because it provides the necessary "locking-mechanism" to hold the core polymerase on the DNA. This enhances the stability and the processivity of DNA polymerase allowing it to synthesize long DNA strands (54). More recent studies show that the beta subunit (DnaN) is required for negative regulation of chromosome replication (see below) and provides feedback signals to the initiator DnaA that the replication fork has been assembled. Part of this thesis describes proteins that may interact with the *Caulobacter crescentus* DnaN.

The DnaN clamp loader mechanism has been well studied in *E. coli*. The delta subunit acts to open the beta-clamp dimer and therefore functions like a 'wrench' (55,56).

Although delta is not an ATPase it derives its energy from conformational changes imposed by the gamma subunits within the gamma complex which exposes delta allowing it to interact with the beta subunit (57). The delta` subunit, may act as a rigid support structure that permits other components of the gamma complex to function (58,59). The final two subunits of the gamma complex are the psi and chi subunits. The chi subunit interacts with SSBs and it is important for the exchange of the primase, at the site of priming, with the clamp loader/DNA polymerase that permits DNA synthesis (60). The role of the psi subunit is not clear however it does interact with gamma and may form a tether between the gamma-complex and the chi subunit (61). The gamma complex essentially works in tandem with the beta-subunit (DnaN) to increase the processivity of the core polymerase (62,63). The steps of clamp loading are as follows; Prior to DNA synthesis the gamma complex facilitates the loading of the beta-subunit onto the double stranded template because the gamma-complex is a DNA-dependent ATPase that recognizes primed DNA (63). The ATPase activity of the gamma-complex is stimulated by the beta subunit directly and subsequently loads the beta subunits which form a dimer ring on the DNA template (54,62).

Chromosome replication also requires topoisomerases. Shortly after the Watson and Crick DNA structure was proposed, it was argued that replication of helical DNA would impose topological constraints, making DNA replication impossible. To address this problem, topoisomerases (swivelases) were suggested as a biochemical activity that could in principle bypass this limitation. In *E. coli*, DNA gyrase is an essential component of chromosome replication, that relieves positive super coil tension created by the moving

replication fork (40,64). As DNA gyrase is a type II topoisomerase, it relieves tension by introducing double strand breaks. DNA gyrase also acts as a sensor and through an ATP-dependent process, relieves DNA tension by introducing negative supercoils in DNA (65).

The DNA Polymerase III holoenzyme also needs other enzymes to complete DNA replication. These include DNA Polymerase I to remove the RNA primer and fill the remaining gap left by the vacated primer, and DNA Ligase, which covalently cross-links the DNA backbone after gap filling (64).

During the elongation phase of DNA synthesis, replication sub-assemblies are brought together and the fork begins moving out from the origin of replication. DnaB, primase and the Pol III holoenzyme work together as a unit with the beta-subunit and DNA Pol III tightly bound to the DNA. In this framework, DnaB, primase, and the gamma complex act distributively, that is they have the ability to associate and dissociate from the replication fork (42). However, a number of complexities exist in terms of lagging-strand synthesis and DNA Pol III remains bound to the replication fork even though it must dissociate from the lagging-strand template after each round of primer extension suggesting it is the protein-protein contacts that ensure the lagging strand polymerase remains associated with the moving replication fork (66). A typical round of Okazaki fragment synthesis consists of the recruitment of the primase to the replication fork followed by synthesis of the primer on the lagging strand. An interaction between primase and the polymerase holoenzyme is assumed because DNA Pol III influences the primer length specified by primase (42,67,68). The interaction between primase and polymerase suggests that the holoenzyme signals for the release of primase (66). In this

manner the distributive activities of the gamma-complex and the beta-subunit can rapidly direct the lagging strand polymerase from the previous Okazaki fragment to the newly synthesized primer. In contrast, leading strand synthesis need only be primed once at the replication origin.

1.1.3 Termination of DNA Replication

Termination, occurs in a region approximately 180° from *oriC* and it is preceded by flanking zones that impede replication fork movement. These termination zones contain ten *ter* sites (TerA-TerJ), highly conserved 23bp consensus sequences, whose arrangement ensures that replication forks can pause inside the termination zones and thereby synchronize their arrival very close to 180° from *oriC* (69,70). The termination utilization substance (Tus) binds all ten *ter* sites in the *E. coli* termination zone (71). The mechanism of termination involves the dissociation of the DnaB helicase by DNA-bound Tus (72,73). DnaB dissociation depends on the orientation of DNA-bound Tus and the opposing alignment of ter binding sites ensures that the movement of both the leftward and rightward replication forks is stalled. The result is that each replication fork stalls in a termination zone and replication fork movements are coordinated to ensure complete duplication of the genome. How Tus unloads DnaB is not clear but current models suggest that Tus acts like a clamp to physically block advancing replication forks (74) or Tus imposes DNA secondary structure that destabilizes DnaB interactions with the replisome (75). It should be noted that mutants of Tus which do not disrupt DNA binding are fully capable of preventing replication fork progression (75,76).

Immediately following termination, the chromosomes are intertwined and must be unlinked (decatenation). This activity is carried out by Topoisomerase (Topo) IV which is recruited by the DnaX subunit of DNA polymerase III within the termination zone. Like DNA gyrase, Topo IV is a type two topoisomerase that promotes a double strand break to resolve the two chromosomes (77). Topo IV is also part of the larger mechanism of chromosome segregation and forms a functional complex with FtsK, a DNA translocase involved in the resolution of chromosome dimers at the septal ring (78). The close proximity of newly replicated chromosomes provides opportunity for site specific recombination and the formation of chromosome dimers (79). Resolution of these dimers is achieved by the Xer recombination system (80). The Xer system is comprised of two site-specific recombinases, XerC and XerD, including two target DNA sites, *dif*, found at the *E. coli* terminus where recombination takes place (80). Like Topo IV, XerC and XerD activity is dependent on the cell division protein FtsK (81). Taken together, FtsK demonstrates a central role in coordinating the terminal events of replication termination (decatenation), chromosome segregation, and recombination at the site of cell division.

1.2 Yeast Replication

1.2.1 The Autonomous Replicating Sequences

In eukaryotes, like we will see for *Caulobacter*, DNA replication is limited to once and only once per cell cycle. The eukaryotic cell cycle is conspicuously divided into G1-S-G2-M phases where chromosome replication is limited to initiating only once per cell cycle. Also, unlike *E. coli* and other bacteria, eukaryotic genomes carry numerous replication origins that have been equated in yeast with autonomous replicating sequences (ARS) (82). The following reviews are restricted to chromosome replication in the budding yeast *Saccharomyces cerevisiae*, because it is the best studied organism in the eukaryotic kingdom.

Similar to prokaryotic origins, ARS elements in yeast house important consensus sequences which include a highly conserved AT-rich domain (A element) (83) that is essential for origin function (84). The A element is the primary binding site for the origin recognition complex (ORC), a multi-subunit protein complex that shares similar functional characteristics with the prokaryotic DnaA protein. ORC, like DnaA, belong to the AAA+ family of proteins because all members of this family share a conserved structure of 220 amino acids (85). Yeast origins (ARS elements) also contain auxiliary binding sites, or B elements, that facilitate ORC binding (86,87), DNA unwinding, and the binding of transcription factors (88). However, looking beyond the phylogenetic horizons of yeast, one rarely finds sequence conservation as a defining feature among the origins of higher eukaryotes (origin flexibility). One hypothesis suggests epigenetic

control (i.e. chromatin dynamics), which considers the packaging of the chromosome as another level of regulation (89). However, the conservation of ORC proteins among all eukaryotes studied suggests they are a common denominator for regulating chromosome replication. While ORC always binds on or more A elements (a vague AT-rich sequence) there is a great diversity of B elements used by yeast ARS elements. This observation helps explain why it is difficult to identify origins by simply scanning the DNA sequence.

1.2.2 Cell Cycle Model of Eukaryotic Replication

There are approximately 400 origins per haploid yeast genome each with its own ARS element. Where tested, these regions are capable of supporting autonomous plasmid replication and, in principle, support the replicator model of replication (90). However, while all yeast origins of replication contain ARS elements, these origins are not uniformly utilized during a given round of replication (91,92). To ensure the precise duplication of chromosome DNA, replication origins are licensed by the loading of the MCM2-7 proteins (discussed below) during mitosis and G1 (93). The license is subsequently utilized and turned off during S-phase and G2 to ensure that no extra rounds of replication are initiated (Figure 4). Eukaryotic licensing is distinct from chromosome replication in *E. coli* because licensing requires the input of positive signals while *E. coli* replication is under negative feedback control. This dichotomy of chromosome replication is important and underlies the major theme of this thesis: How do cells limit DNA replication to once per cell cycle?

1.2.3 Origin Recognition Complex (ORC)

An analysis of the *S. cerevisiae* origin recognition complex (ScORC) reveals that coordination of DNA replication is primarily concerned with controlling the activity of ScORC. ScORC is the principle initiation factor that recognizes and binds to the ARS element, possibly forming a six membered (ScORC1-6) protein ring around the DNA (94). In the general model, ORCs bind ARS DNA and recruit replication factors (95). As mentioned previously, ScORC recognizes and binds the A-element of the ARS and it is believed subunits ORC1p, ORC2p, and Orc4p contact the DNA template (96). DNA recognition requires ATP binding to ORC1p and ORC5p. The stability of ORC1p-ATP complex is dependent on the availability of functional ORC DNA binding sites. This suggests ATP binding to Orc1p is dependent on a functional interaction between fully assembled ScORC its cognate DNA binding sites (97). Analysis of ScORC/DNA complexes *in vivo* reveals that ScORC occupies origin DNA continuously (98,99) and two types of ScORC/DNA complexes have been identified, a pre- and post-replicative form. Genomic foot printing of the post-replicative state suggests only ORC is bound to the origin DNA and is not sufficient to stimulate DNA replication on its own (100). At the end of mitosis and throughout G1 the pre-replicative state is characterized by a region that extends and overlaps the ORC footprint demonstrating that at least two steps are required in the assembly of replication complexes at yeast origins (100).

1.2.4 Components of Replication Initiation

ORC proteins are essential for chromosome replication as disruptions or mutations of ORC subunits are lethal because they reduce the efficiency of origin firing and lead to cell cycle arrest (101,102). The major interacting partner of ScORC is the ATPase ScCdc6 required for replication initiation but not elongation (103). The ATPase function of ScCdc6 is an essential requirement for DNA replication (104) and suggests that the ScCdc6 ATPase activity couples protein conformational changes in ScORC to facilitate the binding of subsequent replication proteins (85). The N-terminal domain of ScCdc6 contains a recognition motif for cyclin-dependent kinases (CDKs) (104,105). The next protein complex recruited to the origin are the MCM proteins which function as the replication helicase (106), similar to the role DnaB plays in *E. coli* (107). This complex of proteins is made up of six subunits (MCM2-7). Deletions in any MCM results in a loss of cell viability (see review: (108)). In *S. cerevisiae*, MCM proteins are dynamically localized to the nucleus in G1 and exported to the cytoplasm during S-phase (109). Once again, ATPase activity is a feature of this complex and is required for translocation along the DNA (110). A unique function of MCM2 is to limit the helicase activity of the assembled MCM complex suggesting that it prevents unwanted helicase activity during the assembly phase of replication initiation (111). Together, ORC, Cdc6, and MCM constitute the pre-replication complex (pre-RC) and it is this complex which enters S-phase and directs replication fork assembly.

1.2.5 Assembly of Initiation

While the molecular mechanism of DNA replication in eukaryotes has not been clearly elucidated, an outline of the steps necessary for replication initiation has nonetheless begun to emerge. Clearly, ORC, Cdc6, MCMs and other accessory proteins (discussed below) have been identified at yeast replication origins and there appears to be an ordered interdependence among these proteins. For instance ScMCM proteins require ScORC and ScCdc6 for functional interaction with the replication origin (98,99). Once MCM has bound to the origin it is no longer dependent on ORC or Cdc6 and this suggests that the major function of these proteins is the recruitment of the MCM complex (112,113). One of the first initiation factors to interact with the pre-RC is Mcm10 which stabilizes the MCM helicase by facilitating its phosphorylation by cyclin dependent kinases (CDKs) (114) and this interaction is important for initiation (115,116). A second role for Mcm10 is the recruitment of Cdc45 (117,118). The stability of Cdc45 with the replication origin also depends on the association of Cdc6 and MCM proteins (98,119). Unlike the other components described, Cdc45 loading is CDK dependent during the G1 to S transition (119). The role of Cdc45 suggests it recruits DNA polymerase alpha to the initiation complex (119-121). The suggestion that Cdc45 associates with the moving replication fork (98,120) along with observations of Cdc45-MCM complexes *in vitro* (122) suggests that Cdc45 acts to tether the MCM helicase with DNA polymerase alpha. Therefore Cdc45 may act similarly to the tau protein which tethers DNA polymerase III with the DnaB helicase in *E. coli* (123).

While eukaryotic DNA replication is inherently more complex than bacterial replication (because of the greater need to control the firing of multiple origins), the components that

make up the replication fork system are homologous. The first component isolated was proliferating cell nuclear antigen (PCNA), so named because its expression significantly increases in rapidly proliferating cells (124). PCNA is functionally analogous to the beta-subunit in *E. coli* and therefore acts as the major clamp for DNA polymerase (125). The yeast clamp loader complex, RFC is a hetero-pentameric AAA+ family that has similar structural and functional characteristics to the *E. coli* gamma complex. Like *E. coli,* the *S. cerevisiae* RFC complex has been shown to load DNA polymerase delta onto primed single stranded DNA in an ATP dependent process (126,127). The trimeric single stranded binding protein RPA (replication protein A) is also found at eukaryotic replication forks and provide a similar role in stabilizing single stranded DNA as SSB in *E. coli* (128). The DNA polymerases that participate in DNA replication are at least three, alpha, delta, and epsilon. Originally, Pol-alpha was considered to be the primary polymerase in eukaryotic DNA replication because it was the first to be biochemically purified and both polymerase and primase activities often co-purified *in vitro* (129,130). Subsequently, DNA Pol-delta was confirmed as the primary replicative polymerase. Pol-delta displays exonuclease proof-reading activity and high processivity when coupled with PCNA (131,132). The third replicative polymerase identified at the replication fork was DNA Pol-epsilon. While Pol-epsilon is also essential, it is not clear whether this polymerase contributes intrinsic replicative activity or provides structural support for components of the replication fork and suggests a role in chromatin remodeling or checkpoint control (133,134). It is currently believed that DNA Pol-delta and Pol-epsilon, in conjunction with PCNA, catalyze both leading and lagging strands synthesis at yeast replication forks.

1.2.6 Termination

Very little information is available regarding the termination of eukaryotic DNA replication but the general notion is that termination in yeast occurs randomly at sites of contact between converging replication forks (135,136). However a similar mechanism to the *E. coli* ter and Tus pathway has been identified in yeast. For instance, ongoing transcription of ribosomal RNA (rRNA) in *S. cerevisiae* directs replication forks towards the same direction as transcription. Subsequently, termination of DNA replication in this region occurs at replication fork barriers (RFB) found upstream of rDNA promoters (137,138). The characteristics of this system ensure that replication forks do not collide with RNA Polymerase I. In fission yeast, mating type switching is regulated by imprinting at the RTS-1 element, a region that is specifically bound by the replication termination factor rtf1p (139). The bound rtf1p-RTS-1 complex blocks replication fork movement and stimulates recombination at the mating type locus (140,141). Therefore, replication termination at this locus is required for genetic recombination, mating type switching, and subsequent cellular differentiation.

1.3 Replication in Archaea

While *Archae* bacteria, with their circular chromosomes and genetic operon organization, appear similar to *E. coli* and other eubacteria, they are evolutionarily distant because their replication, transcription, and protein synthesis genes are strikingly similar to those of eukaryotes. The replication origins of *Archaea* vary in length from 100 to 1000 bp (142) and in one study were found to be flanked by the homologs of eukaryotic ORC/Cdc6

replication genes (143). Replication is believed to be bidirectional from a single origin, however multiples origins have been recently suggested for *Methanocaldococcus jannaschii* (144) and *Halobacterium halobium* (145) although it is not known whether these putative origins are utilized simultaneously during the same cell cycle. Homologs of all of the classical components of eukaryotic replication have been isolated such as ORC, Cdc6, and MCMs and components of the replication fork, PCNA, RPA, RFC, primase, and two DNA polymerases (PolB, PolD) (see review (146)). Therefore, *Archaea* apparently use a eukaryotic replication system within a bacterial chromosome context.

2. Restricting chromosome replication to once per cell cycle

The primary means of regulating DNA replication is to control replication initiation through the coordination of the ordered assembly of replication proteins. As expected, mechanism are available to control both the pre-replicative and post-replicative states of replication to ensure 1) that replication is limited to once per cell cycle and 2) that replication is coordinated with other cellular processes (e.g. cell division). Checkpoint controls are then utilized to coordinate cellular processes with chromosome replication.

2.1 Controlling replication in E. coli

While checkpoint controls remain to be elucidated, there has been much progress in understanding the negative feedback mechanisms that restrict chromosome replication.

As described above in section 1.1.1, *E. coli* replication depends on DnaA to recognize, unwind, and prepare *oriC* for replication. However, once replication has initiated the cells must ensure that the progeny of division only receive a single chromosome. Therefore extra rounds of replication must be prevented during each cell cycle. In *E. coli* , three mechanisms; sequestration, titration, and Regulated Inactivation of DnaA (RIDA) are responsible for preventing extra chromosome replication by directly controlling DnaA activity and/or its accessibility to *oriC* (Figure 5).

2.1.1 Sequestration of *oriC*

The *E. coli oriC* is bound and repressed by a process termed "sequestration." Sequestration requires the SeqA protein and the Dam methyltransferase. Prior to replication, both strands of the chromosome are methylated at adenine residues found within the dam methylation sequence GATC (9,147,148). During replication, hemimethylated DNA is generated, because the newly synthesized DNA strands are not yet methylated. Re-methylation by Dam is rapid (> 1min) at most chromosome sites. However, at *oriC*, which contains 11 GATC methylation sequences, remethylation takes as much as a third of the cell cycle (~20 min) to complete (149). Hemimethylated origins are not competent for replication *in vivo* (150), although they are suitable substrates *in vitro* (151,152). The discrepancy is due to the activity of SeqA (153,154) which recognizes and preferentially binds hemi-methylated DNA (155,156). SeqA has also been shown to sequester origins at the cytoplasmic membrane and suggests a direct mechanism for blocking the reassociation of DnaA with *oriC* (11). This eclipse period, which lasts approximately 10 minutes in WT *E. coli* is significantly reduced (5 min) in

SeqA mutants (153). The reason for over initiation in *seqA* strains may be due to rapid re-methylation in the absence of SeqA or the to restrict the accessibility of other replication proteins such as DnaA (157).

2.1.2 Titration of DnaA

The second mechanism for restricting replication initiation uses the DnaA titration locus *datA*. This region of approximately 1kb located at 94.7 minutes (near *oriC*) on the 100 minute *E. coli* genomic map is tightly bound by DnaA (158,159). The *datA* locus contains four high-affinity DnaA consensus binding sites that can bind approximately 300-400 DnaA molecules. As this region is close to the *oriC* the titration potential of *datA* doubles shortly after the start of chromosome replication. Deletion of the *datA* locus results in over-replication and suggests that *datA* functions to limit replication initiation by reducing the number of DnaA molecules available to bind *oriC* DNA.

2.1.3 Regulatory Inactivation of DnaA

The predominant mechanism preventing over-replication in *E. coli* is termed Regulated Inactivation of DnaA (RIDA) (160). A recent comparison of RIDA, sequestration, and titration revealed that only cells lacking functional RIDA over-initiated chromosome replication. This apparent contradiction in the literature is not easily explained, but may result from strain variation. The necessity for and mechanism of RIDA were originally suggested by the temperature sensitive DnaAcos mutant that hyper-initiated chromosome replication at the non-permissive temperature. This DnaA mutation had low ATP hydrolysis activity implying that switching between DnaA-ATP and DnaA-ADP forms

was an important regulatory principle. From these subsequent studies two major components were identified, the replication fork clamp (beta subunit) (161) and a small soluble member of the AAA+ family, IdaB, that inhibited replication in reconstituted *in vitro* assays (162,163). The *idaB* gene was subsequently identified and renamed Hda (Homolog of *dnaA*) (164,165). Disruptions of Hda produced cells with multiple chromosomes suggesting that Hda affects initiation *in vivo* (164,166,167). The mechanism by which Hda and the beta-clamp function is currently speculative, but Hda, which is homologous to the ATPase domain of DnaA, stimulates the weak intrinsic ATPase activity of DnaA. This suggests that Hda is a nucleotide switch factor that recognizes and stimulates conversion of DnaA-ATP (active) to DnaA-ADP (inactive). The role of the beta-clamp, an obligate component of replisome assembly, implies that successful assembly of the replisome is an important negative-feedback mechanism that inhibits DnaA activity. The recent observation that the beta-clamp and Hda colocalize *in vitro* suggests the beta-clamp stimulates or stabilizes Hda activity and also that the beta-clamp brings Hda to DnaA (168). In addition, DnaA recruits DnaB helicase (31) suggesting a secondary role for DnaB in the RIDA mechanism. A speculative RIDA function for DnaB might be to destabilize the DnaA oligomer through local changes in the structure of the open DNA complex.

In summary, *E. coli,* has at least three mechanisms that ensure chromosome replication is restricted to once per cell cycle. These negative feedback mechanisms ensure that superfluous rounds of chromosome replication are prevented.

2.1.4 Other Checkpoints

2.1.4.1 Response Regulators and Two Component Signaling

Two-component systems define a broad range of regulatory systems that permit bacteria, and other organisms, to quickly adapt to changing environmental conditions (169). Each two-component system consists of a histidine protein kinase sensor (HPK) coupled to a response regulator (RR). In general, external stimuli activate the pathway by modulating HPK activity (Figure 6) leading to autophosphorylation at a conserved histidine residue. The RR is subsequently recruited to catalyze a phospho-transfer reaction with the HPK-phosphate becoming transferred to a conserved aspartate within the regulatory domain of the RR. This is followed by activation of an adjacent effector domain and execution of the specific response. The intrinsic phosphatase activity of the RR ensures the response is limited lasting seconds to hours. The current model suggests that the level of RR phosphorylation is the ultimate signal determining the output response (170). Two component systems (TCS) are ubiquitous and account for nearly 1% of the encoded proteins in a given species of eubacteria (169). TCS are involved in many aspects of bacterial cell growth; from chemotaxis and virulence to osmoregulation and oxygen stress (169). However, two component systems are also involved in cell cycle regulated processes such as chromosome replication and differentiation and section 4 of this literature review will describe in detail the role of CtrA a response regulator that plays a significant role in the *Caulobacter* cell cycle.

Perhaps the best studied two component system is the NR_I/NR_{II} pathway that controls nitrogen regulation in *E. coli.* In response to nitrogen starvation, the HPK, NR_{II}, phosphorylates the RR, NR_I (171). Phosphorylated NR_I binds to sequences upstream of the nitrogen-regulated promoters and stimulates sigma-54 dependent gene transcription of the *glnA ntrCB* operon. The promoter region of this operon contains three strong NR_I-binding sites (172,173) and NR_I or NR_I -phosphate bind equally well to these sites (174). However, occupation of NR_I at these sites does not stimulate transcription. Only NR_I-phosphate is able to activate transcription because phosphorylation of NRI increases the cooperativity constant, a measure of dimer formation. The imposed conformational changes of the dimer-bound DNA stimulates transcription by allowing dimers at adjacent sites to communicate and form tetramers (174). This suggests 1) that NR_I binding to DNA is not the primary signal for transcriptional activation and 2) that phosphorylation is not a signal by itself but provides the necessary conformational changes (dimer/tetramer formation) required for NR_I-mediated transcription. It has also been demonstrated that low concentrations of NR_I-phosphate, which freely form dimers, cannot stimulate transcription (175). Only when a critical concentration of NR_I-phosphate dimers has been achieved (> 50 μM) can tetramers form and drive the conformational changes required to activate transcription. The mechanism of NR_I provides a basis for Chapter II where the role of phosphorylation and DNA binding of the response regulator CtrA will be considered.

2.2 Replication Licensing in Yeast

2.2.1 Kinases

Replication initiation in *S. cerevisiae* relies on the essential activities of cyclin-dependent kinases to limit replication to once per cell cycle. In particular, two kinases, CDK (ScCdc28) and Cdc7 act at different stages of the cell cycle to control replication. In general, the catalytic activity of CDKs depends upon association with cyclins (see review (176)) and it these cyclin-CDK hetero-complexes that help coordinate the initiation of replication (177,178). CDK activation is established during late G1 and achieves maximal activity during S-phase and is necessary to prevent re-replication (93). The B-type cyclins ScClb5 and ScClb6 are responsible for triggering S phase. However mutations that disrupt these cyclins can be rescued by other B-type cyclins demonstrating a functional redundancy within this family (179).

A primary target of S-phase CDK-cyclin signaling is Cdc6, a protein which promotes replication (104,180). The role of this interaction has been extensively studied with the Cdc6 homolog of *S. pombe*, Cdc18. The recruitment of CDK, by Cdc18, to the replication origin stimulates CDK phosphorylation of Cdc18 (181). Phosphorylation of Cdc18 apparently inhibits its activity because mutants that abolish CDK-dependent phosphorylation show increased Cdc18 activity as evidenced by over-replication (181,182). Therefore, hyper-phosphorylation of Cdc18 in early S-phases helps to limit replication initiation.

The MCM proteins are important targets of CDKs, but the function of this phosphorylation is not clear. Phosphorylation may stimulate MCM activity early in S

phase or it may help to limit MCM activity after initiation. In *Xenopus laevis*, phosphorylation of MCM4 in mid S-phase coincides with the dissociation of MCM complexes from the chromatin, suggesting phosphorylation limits MCM activity after initiation (183-185). In *S. cerevisiae*, chromatin immunoprecipitation (ChIP) experiments have demonstrated MCM interactions with CDKs at the replication origin *in vivo* (99). It is clear however that down regulation of CDK activity during G2 results in over-replication during the *S. cerevisiae* cell cycle and suggests MCM proteins are able to reload during G2 in the absence of CDK activity (186). The model suggest that declining levels of CDK during late mitosis and G1 permit licensing while increasing CDK activity in late G1 prevents further licensing and promotes the initiation of chromosome replication (187).

ORC proteins are also targets of CDK activity (102,188). ScORC remains associated with the chromatin throughout the cell cycle (99,189). Since phosphorylation does not change ORC binding to DNA, what affect phosphorylation has on ORC activity is not yet clear. CDK recruitment to the replication origin may also provide important remodeling signals needed to control the replication potential of ORC, Cdc6, or MCM. Cdc45 binding to the replication origin is also CDK-dependent (119) but there is no evidence that this is a direct result of Cdc45 phosphorylation by CDKs.

The second CDK responsible for regulating replication is the Cdc7-Dbf4 kinase (190). ScCdc7 is a constitutively expressed serine/threonine kinase predominantly active during S and G2 (191). ScCdc7 activity is controlled by the regulatory activity of the Dbf4 subunit (192). Dbf4 is also important for targeting Cdc7 to the replication origin and also

depends on intact ORC binding sites for association (193). *S. cerevisiae* Mcm2, 3, 4, and 6 are substrates for Cdc7 *in vitro* (194). As opposed to Cdc6, phosphorylation of MCMs by Cdc7 suggests a positive role for stimulating helicase activity (195). Since Mcm2 inhibits MCM helicase activity it is believed that its phosphorylation by Cdc7 may adjust the activity of the MCM complex (111).

In eukaryotes, kinases regulate the subunit assembly of protein complexes at replication origin. While the molecular mechanisms have not been worked out, it is clear that CDKs function as oscillators to control the steps of replication and ensure replication occurs only once. As we will see in Chapter III, a different class of "switch factors" plays an important role in bacterial replication origins as well.

3. The Role of Protein Chaperones in DNA replication

A protein chaperone alters the activity of other protein(s) through protein-protein contacts that either guide or redirect the folding or aggregation pathways of the target protein. Therefore, chaperones act as stage conductors to control the functional fate of other proteins. Chaperones have an established role in controlling phage and plasmid replication. The *E. coli* chaperones ClpX and DnaK also target numerous proteins for degradation. The consequences are that chaperones modulate the activity occurring at the replication origin. The following literature review shows that chaperones can be used in

diverse ways to promote replication. I will later propose that chaperone ClpX has a new role at the *Caulobacter crescentus* chromosome replication origin.

3.1 Phage Replication

3.1.1 Lambda (λ) Phage

During the lytic phase of lambda phage development, replication requires the utilization of viral and host encoded proteins to synthesize viral genomic DNA. The major viral proteins are the initiator λ-O protein which binds to ori-lambda (196) and recruits the λ-P protein in complex with the DnaB helicase (197) forming a pre-primosome complex. This complex represents the final stage of replication initiation in lambda phage as the removal of the lambda P protein stimulates DnaB helicase activity and the loading of the replisome (198). However, the O-P-DnaB complex is also very stable and chaperone activity is needed to release DnaB. RNA polymerase is also a requirement for lambda replication, presumably by assisting in template unwinding (199,200).

3.1.1.1 DnaK Chaperone

DnaK, DnaJ, and GrpE work together to provide a number of important biochemical functions (201). For instance, temperature sensitive mutations in any one of these components blocks λ phage growth (202,203). The DnaK chaperone complex functions to release DnaB helicase from an unusually strong bond with λ-P at ori-λ origins of replication (204). Looking at the specific components of the DnaK chaperone complex we find the chaperone DnaK, a conserved member of the Hsp70 family of heat shock proteins (205). In other contexts, DnaK functions as an unfoldase, carrying with it weak

intrinsic ATPase activity and the recruitment of DnaJ and GrpE are required to increase ATPase activity several fold (206). While DnaJ is a bonafide chaperone and prevents protein aggregation (205), it is not clear whether this activity is enhanced when complexed with DnaK (207). Unlike DnaJ which is dispensable, GrpE is essential for *E. coli* viability. GrpE acts as a nucleotide exchange factor for DnaK (206,208) and also assists in the release of polypeptides from DnaK. In terms of λ phage replication, DnaJ is the first chaperone to recognize and bind the pre-primosome complex with DnaK and GrpE following (209). In this manner, the DnaK chaperone complex relieves the inhibition on DnaB by "rearranging" λ-P activity. λ-P has been shown to remain bound to the O-some after DnaB release and loading on the λ replication origin.

3.1.1.2 The ClpX/ClpP Chaperone/Protease Complex

During the initial stages of *λ* phage replication, the O protein recognizes four iterative sequences in *oriλ* forming a specialized nucleoprotein complex (O-some) that serves as a scaffold structure for the remaining assembly reaction (210). The unbound O protein is generally unstable because it is a target of ClpX/P mediated degradation. Mutations in either complex abolish λ-O proteolysis (211). However, it has been demonstrated the λ–O p/rotein is protected from proteolysis when bound to DNA and suggests that ClpX/P functions to titrate out free λ-O protein. While the absence of ClpX/P does not influence λ growth there has been evidence to suggest ClpX/P is important for the lysis-versus-lysogeny switch in slow growing bacterial cells (212). Whatever role ClpX plays in λ phage development, it should be noted that λ-O was one of the first proteins where the stepwise mechanism of ClpX unwinding was elucidated *in vitro*. These studies show that

ClpX contacts a dimer of λ-O followed by a systematic unfolding and translocation into the lumen of the ClpP protease (213,214).

3.1.2 Mu Phage

Mu phage replicates its genome through a chain reaction of genomic transposition events (215). The minimum components required are the phage encoded MuA transposase and ClpX chaperone mediated regulation of transposase activity. The steps of Mu transposition include 1) the recognition of Mu genomic DNA by tetrameric MuA; 2) recruitment of new target host DNA by the accessory protein MuB 3) cleavage of the Mu genome by MuA; 4) strand transfer between donor and target DNA strands; 5) DNA replication (216). The progression of Mu transposition results in a stepwise increase in the stability of the MuA-DNA complex and therefore replication of Mu genomic DNA, which requires MuA protein displacement is inhibited (217). Paradoxically, MuA is also required to promote replication of Mu DNA and it is the disaggregating activity of ClpX that permits replication (218). It should be highlighted that ClpX resolution of MuA-DNA complexes occurs independently of ClpP protease, and that ClpP is dispensable for Mu growth (219). ClpX does not mediate the release of MuA but rather ClpX destabilizes its contact with DNA (220) by targeting one of the subunits from the MuA tetramer complex (221). This new configuration of MuA then permits Mu DNA replication to proceed by stimulating the recruitment of the replication complex. Although ClpX is generally regarded as a protein unfolding machine that docks with ClpP, this example clearly shows that ClpX can act alone to perform a very precise protein remodeling reaction.

3.1.3 Bacteriophage P1

P1 replicates by forming a plasmid circle during its lysogenic phase. P1 replication requires a phage encoded RepA protein to bind sequences found within the P1 origin (222). Dimers of RepA alone or in complex with DnaJ can bind the P1 origin however with low affinity. The addition DnaK increases the binding affinity of RepA-DnaJ complexes to *oriP1 in vitro* by stimulating the monomerization of RepA (223). This monomerization by DnaJ and DnaK can be functionally replaced by the chaperone ClpA. ClpA is a primary chaperone for the targeting of substrates for proteolysis through the ClpP protease (224,225). In regards to P1 replication, only ClpA chaperone activity, independent of ClpP (226), is required to stimulate RepA monomerization.

3.2 Plasmid Replication

3.2.1 RK2 Plasmids

RK2 is a broad-host range plasmid in Gram-negative bacteria (227). RK2 plasmids minimally require two plasmid encoded components for replication, the replication origin (*oriV*) and the Rep protein TrfA (228,229). The remaining elements of RK2 replication are host encoded and include DnaA, the replisome, etc. (230-232). Replication initiation requires the binding of TrfA to iterative sequences within oriV. The protein aggregation state of TrfA is an important requisite for activation of RK2 plasmid replication (233). The chaperone ClpX, stimulates TrfA activation *in vitro*, by converting the inactive

dimers to active monomers (234). However, ClpX is dispensable for RK2 maintenance *in vivo* and this suggests other chaperone systems can functionally replace ClpX in this versatile broad-host range plasmid. Of central importance to this thesis is the observation that ClpX chaperone activity can occur independently of ClpP. Therefore, RK2 plasmid replication provides another example where ClpX chaperone activity is functionally separated from its originally proposed role in proteolysis.

3.2.2 RK6 Plasmids

More recent work has also found a role for the DnaK-DnaJ-GrpE chaperone system in activation of the pi initiator protein during the replication of R6K plasmids. The R6K plasmid contains three replication origins (alpha, beta, gamma) and the pi protein, in conjunction with host encoded DnaA, are required to initiate replication from ori-alpha and ori-gamma, while ori-beta seems only to require pi protein for activation. The monomeric form of pi protein is active and the chaperone system must resolve the inactive dimers of pi into active monomers and this activity seems to be required for ori-gamma-specific replication. The role of monomeric pi protein in stimulating replication at ori-alpha and ori-beta is not clearly understood since dimeric pi protein may also be involved in stimulating replication at alpha and beta.

The preceding examples show that phage and plasmid replication requires the activity of protein chaperones. In all cases this chaperone activity is not coupled to protein degradation. A recent review by Burton and Baker proposes that proteins targeted to

chaperone/protease complexes may, under special conditions, be refolded instead of degraded (216). This extends the role of chaperones to that of "quality control" and suggests wider regulatory roles. Much less is known about how chaperones influence chromosome replication, a key topic of this thesis.

4. *Caulobacter crescentus*

This bacterium provides our model for chromosome replication. *Caulobacter crescentus* is a Gram-negative bacterium that displays a unique asymmetric pattern of development (for review see (235)). This organism is distinguished by a cell division program which yields two morphologically distinct progeny each with its own unique cellular program of chromosome replication (Figure 7). One of the obvious anatomical features of *Caulobacter* is the crescentoid shape of its membranes. This is achieved, in part by a helical polymer referred to as crescentin, a homolog of intermediate filaments found in eukaryotes (236). Each round of cell division produces a flagellated and chemotactic swarmer cell and a sessile stalk cell. In swarmer cells, DNA replication is selectively blocked and swarmers must undergo programmed cellular differentiation to the stalked cell type in order to initiate DNA replication. Therefore, *Caulobacter* represents a useful model to study how DNA replication is coupled with cell growth and development and to uncover the mechanisms that limit chromosome replication within its cell cycle.

4.1 The Caulobacter Cell Cycle

Caulobacter cell division releases a non-replicating swarmer cell with a single polar flagellum, pili, and chemotaxis apparatus (237). After a prescribed period, the swarmer begins differentiation to the stalked cell (238). This transition stage event includes the ejection of the single flagellum, the synthesis of the stalk appendage, the retraction of pili, and the degradation of chemotaxis proteins. This stage also marks the initiation of chromosome replication because chromosome replication only occurs in the stalked cell. As chromosome replication proceeds, a new polar flagellum is synthesized opposite the stalk pole (old pole) and the growing cell elongates, thus defining the swarmer compartment (new pole) and the stalked compartment of the pre-divisional cell. Newly synthesized daughter chromosomes are segregated to the poles of each compartment and cytokinesis generates a new replication restricted swarmer and the regenerated stalk cell immediately enters a new round of replication and cell division. The cell cycle behavior of *Caulobacter* represents a sophistication typically attributed to eukaryotic cells and in this way, stalked cells are likened to stem cells as they continuously synthesize and develop new swarmer offspring. Therefore, the *Caulobacter* cell cycle helps us to pose specific questions regarding replication control.

4.1.1 Temporal Control and Transcription

The sequencing of the complete *Caulobacter* genome (239) permitted the investigation of the global genetic networks controlling the *Caulobacter* cell cycle. Of the estimated 4000 open reading frames identified in the *Caulobacter* genome, approximately 20% of the corresponding RNAs were cell cycle regulated (240). A subsequent proteomic

analysis revealed that protein abundance coincides with fluctuating mRNA levels demonstrating that protein abundance correlates with gene transcription (241). For instance, genes required for initiation of DNA replication are synthesized in swarmer cells and during the swarmer to stalk transition (G1-S). Genes encoding the replisome and nucleotide biosynthesis are also expressed early and are followed by genes encoding chromosome segregation and cell division proteins in the predivisional cell (240). Temporal synthesis of proteins during the cell cycle is also coupled to the degradation of these proteins and permits the staged removal of components whose role in the cell cycle has been completed (242-244). As we will see, chaperones also play an integral role in the control and periodicity of various activities within the *Caulobacter* cell cycle.

The *Caulobacter* cell cycle is controlled by an oscillating genetic switch comprised of the response regulator CtrA and the transcriptional regulator GcrA (245) each controlling a distinct classes of proteins required for cell cycle progression. CtrA is a master regulator (discussed below) that controls the transcription of numerous cell cycle genes and also plays a role in negatively regulating chromosome replication in swarmer cells (246,247). The newly identified GcrA regulator is a positive transcriptional regulator of the initiator DnaA as well as for genes important for the maintenance of asymmetry (248). The oscillatory nature of the CtrA/GcrA network puts the two proteins out of phase because GcrA positively regulates CtrA while CtrA represses GcrA, defining an important temporal and spatial division in the *Caulobacter* cell cycle.

4.1.2 Spatial Control and Polar Development

Spatial control in *Caulobacter* defines a group of activities that control the subcellular address of various proteins required for the synthesis of structures important for *Caulobacter*'s morphological development. Of primary interest is the polar localization of kinases that coordinate the biosynthesis of pili, flagella, and the stalked appendage. Therefore, asymmetry in *Caulobacter* is achieved through cell cycle-regulated activities that control polar morphogenesis. Polar development in *Caulobacter* is under the control of the histidine protein kinases (HPKs) PleC and DivJ as well as the response regulator DivK (249). All of these components display a "dynamic" pattern of localization (250-252). PleC, which regulates aspects of flagellum biosynthesis, pilus formation and stalk biogenesis (253), is preferentially localized to the flagellar pole in predivisional and swarmer cells and diffuses to the cytoplasm in the stalked cell. DivJ on the other hand controls stalk biogenesis and plays a role in cell division. The localization pattern of DivJ depends on PleC which brings it to the stalk pole in the G1-S transition and dissociates in newly divided swarmer cells. The final component, DivK is primarily cytoplasmic but does show a modest colocalization with DivJ at the stalk pole becoming resolved to both poles prior to cell division. The subcellular address of these developmental factors depends on a hierarchy that sees PleC responsible for the localization of DivJ that in turn controls the polar positioning of DivK. To complete this spatial regulatory loop, PleC controls the dissociation of DivK from the flagellated pole in swarmer cells (250,252). Therefore, an important question regarding this developmental choreography is; what are the signals required to coordinate this process? A closer look at pili development addresses this consideration. The major pilus subunit

PilA is synthesized prior to cell division and assembled at the flagellar pole (254,255). The assembly reaction utilizes a secretory apparatus and the CpaE secretory component has a localization pattern that resembles PleC. CpaE release from the flagellated pole depends on PleC and suggests that recruitment and release of pili biogenesis factors is PleC-dependent since PleC mutants fail to assemble PilA and therefore lack pili (253). PleC placement is dependent on PodJ and mutants of PodJ fail to localize PleC to the swarmer pole or eject the flagellum during the swarmer to stalk transition (256,257). PodJ is unique because it is synthesized as a full length molecule that localizes to the incipient swarmer pole of the predivisional cell and presumably acts to recruit PleC. PodJ is subsequently processed to a smaller isoform that remains with the flagellated pole and its role in stalk biogenesis suggests it might act as a localization factor once the flagellum has been ejected. In this way, PodJ acts as the major regulator of the polar development cascade.

4.1.3 Checkpoint Control

There are at least three major checkpoints in *Caulobacter* all of which are coupled to cell division. These checkpoints monitor the staging of major synthesis reactions and only permit cell cycle progression when these events have properly initiated. These reactions include flagellar biosynthesis, chromosome segregation and DNA replication (discussed in section 4.2). From a global perspective, the cell utilizes checkpoints to monitor the integrity of its membranes, chromosomes, and the positioning of the nucleosome so that cells may be permitted to grow and divide.

4.1.3.1 The Flagellar Biosynthesis Checkpoint

The genes involved in flagellar biosynthesis are organized into four classes (I, II, III, IV) Class I genes initiate the cascade and trigger the transcription of Class II genes during mid S-phase. Class III genes are not transcribed unless all Class II genes are expressed. Similarly Class IV genes are not transcribed or translated and depend on Class III expression. The primary transcriptional regulator is the Class I response regulator CtrA (Chapter II) that controls expression of Class II genes involved in further transcriptional regulation and basal body formation within the inner membrane. Checkpoint control of flagellar biosynthesis is integrated within the Class I and Class II genes (258), since mutations in these genes produce elongated cells, a typical feature of cells that delay cell division. Two Class II genes are responsible for "sensing" the state of flagellar assembly, they are the transcriptional regulator FlbD (259) which stimulates the expression of Class III expression, and the non-structural assembly factor FliX (260,261). The basic model suggests that FliX acts an inhibitor by interacting directly with FlbD (262) and upon completion of early flagellar assembly; FliX releases FlbD allowing Class III expression. New evidence demonstrates that the FlbD/FliX complex communicates directly with the cell division machinery because late stage cell separation is specifically blocked in FlbD mutants (263). These data suggest a direct link between flagellar assembly (activation of FlbD) and cell division.

4.1.3.2 Chromosome Segregation

The asymmetrical cell poles of *Caulobacter* suggest a distinct mechanism is required for chromosome segregation. Chromosome segregation requires at least three proteins

namely, ParA/ParB, and the actin homolog MreB. In stalk cells the origin of replication (*Cori*) is localized to the cell pole and earlier studies suggest the importance of ParA (ATPase) and ParB (DNA binding protein) in anchoring *Cori* to the cell poles since both subunits localize to *Cori* and are essential for viability (264). The disruption of ParA or ParB result in obvious cell division defects suggesting that ParB acts as cell division checkpoint to ensure proper segregation and spatial targeting of the replicated chromosomes (265).

Shortly after replication has initiated, one of the newly replicated origins is rapidly translocated to the opposite pole (266) and the recent discovery of the actin homolog MreB suggests this filament-forming protein may represent the prokaryotic equivalent of the mitotic apparatus (267). The characteristic structure of MreB *in vivo* is the formation of helical cables along the inner membrane of all bacterial species studied (268-270). The location of MreB suggests it has both a role in membrane organization as well as chromosome segregation and in *Caulobacter*. MreB is important for cell polarity including the localization of *Cori* to the cell poles (271). Using a reversible small molecule inhibitor (A22) to block MreB polymerization, newly replicating chromosomes failed to segregate (272). However, administering A22 after *Cori* segregation had taken place did not interfere with subsequent segregation of the remaining chromosome. This suggests that an early MreB-dependent segregation event is followed by MreB-independent segregation of the remaining chromosome. Consequently, MreB apparently interacts with chromosomal regions that are proximal to *Cori,* suggesting this region may form a bacterial centromere (273).

4.2 Chromosome replication

This literature review comes full circle with an examination of DNA replication in *Caulobacter crescentus*. *Caulobacter* replication differs in many respects from *E. coli* replication because it is restricted to a particular cell type (the stalked cell). A recent genome search, by this author, for homologues of *seqA* and a possible *datA* titration locus were unsuccessful. These results suggest *Caulobacter* may not utilize sequestration and titration or that these functions are carried out by other, less obvious, mechanisms. Also, *E. coli* replication is restricted to once per cell cycle through mechanism that directly or indirectly regulate DnaA. I hypothesize that *Caulobacter* uses two separate mechanisms to control chromosome replication. One, that limits the activity of the negative regulator CtrA at *Cori* and a second that restricts the activity of DnaA. As well, the genetic structure of *Cori* is distinct from *oriC* and suggests novel mechanisms are at work to regulate chromosome replication in this dimorphic organism.

4.2.1 The *Caulobacter* replication origin (*Cori*)

Critical to the study of chromosome replication in *Caulobacter* was the identification of a cloned replication origin that supported autonomous plasmid replication, and whose replication was restricted to the stalked cell (274). Bidirectional replication initiates from a unique chromosomal locus (Figure 8) which spans a 1.6 kb region between *hemE* and *Duf299*. (275). Using deletion analysis of *Cori* elements in autonomous plasmid assays, this region was further reduced to 500 bp (276). The *Caulobacter* origin of replication is

distinct from the *E. coli* origin (see Figure 2) and represents a new class of replication origins among the alpha-proteobacteria (277). For example *Cori* will not support autonomous replication in enteric bacteria (277). As well, *Cori* contains only a single conspicuous DnaA box that closely matches the five DnaA boxes found in *oriC* of *E. coli.* The most conspicuous and distinct feature of the *Caulobacter* origin is the presence of five separate binding sites for the response regulator CtrA.

An analysis of *Cori* identifies at least six provisional elements that consist of protein binding sites, transcriptional promoters, and iterative sequences whose biochemical functions are unknown. The first such element is the weak transcriptional promoter (Pw) which directs the synthesis of the *hemE* gene and is absolutely required for autonomous plasmid replication (276). In *E. coli* transcription promotes *oriC* plasmid replication but is not an essential requirement for either plasmid or chromosome replication (36,278). *oriC* is flanked by *mioC* and *gidA* and includes the single *gidA* promoter (see Figure 1) . This contrasts with *Cori* which is flanked by *Duf299* and *hemE* and contains three promoter elements. The second element denotes a 40 bp AT rich region and signifies the location of a strong promoter (Ps) and two CtrA binding sites a and b. This region is dispensable on the chromosome but not for autonomous plasmid replication (235). The third element defines a unique purine-rich stretch whose disruption does not support chromosome or plasmid replication (235) and whose role is not clear, however purine-rich domains have been shown to be important for viral-mediated replication (279). The fourth element defines CtrA binding site c overlapped by an IHF (DNA bending) binding site and deletions in this region are tolerated. Element five represents CtrA binding site d while Element six corresponds to CtrA binding site e adjacent the single DnaA box (274).

In *E. coli,* transcription promotes *oriC* replication (280). Transcription promotes replication by altering the replication origin, presumably by providing negative supercoiled DNA that aids the unwinding of the AT-rich region (36,37). In *E. coli,* the *gidA* promoter adjacent to *oriC* can be functionally deleted without disrupting replication. However, this is not true for the Pw promoter in *Cori* whose disruption does not support chromosome replication (235). The role of the strong promoter (Ps) appears to serve a secondary role since its deletion is tolerated in the whole chromosome but leads to promiscuous replication when disrupted on plasmids (276). The Ps promoter is repressed by CtrA in swarmer cells but is uncoupled from *hemE* expression. This suggests that Ps acts as a timing element to promote replication when Ps activity is derepressed during the swarmer to stalk transition.

4.2.2 Cell Cycle Regulation of Replication

CtrA is a transcriptional regulator and one of the key links between DNA replication and cell cycle progression. CtrA is responsible for controlling the expression of nearly 20% of the cell cycle regulated genes in *Caulobacter.* CtrA activity is regulated by; 1) Transcription, 2) Proteolysis, and 3) Phosphorylation. CtrA auto-regulates its own transcription (281) in conjunction with the newly identified GcrA protein (248). The second mechanism of CtrA regulation is protein turnover which is coordinated by the chaperone/protease pair ClpX/ClpP (282,283). The third mechanism, common to all response regulators, is periodic phosphorylation at a conserved aspartate residue

(247,282,284). Arguably, phosphorylation is the dominant mechanism controlling CtrA activity as both transcription and proteolysis can be circumvented without disrupting cell cycle progression. CtrA~P levels fluctuate during the *Caulobacter* cell cycle. It has been proposed that this cell cycle pattern of phosphorylation alters CtrA affinity for *Cori* DNA providing a primary basis for replication control.

4.2.3 The Initiator DnaA

Caulobacter DnaA is essential for chromosome replication and its homology to *E. coli* DnaA presumes as similar function at *Cori*. Depletion of DnaA blocks new rounds of DNA replication but not ongoing replication, providing evidence that DnaA functions in DNA replication initiation (285). *Caulobacter* DnaA is unique because it demonstrates a specific cell cycle pattern of ClpP-dependent proteolysis (286). Proteolysis of DnaA is biphasic and displays a higher rate of turnover in the non-replicating swarmer cell. At the swarmer to stalk transition, DnaA protein becomes increasingly stable, and suggests this activity maybe under checkpoint control. Presumably the stability of DnaA in stalk cells permits the accumulation of DnaA and the subsequent triggering of replication initiation. The temporal fluctuation in DnaA stability underlies a unique regulatory mechanism in *Caulobacter*. While homologs of the *E. coli seqA*/sequestration and *datA*/titration have not been identified, it is possible that in *Caulobacter*, CtrA may provide a sequestration function in swarmer cells while DnaA proteolysis may represent a form of titration. The third mechanism, RIDA, will be discussed in more detail in Chapter IV.

4.2.4 DNA Methylation and *ccrM*

DNA methylation in *Caulobacter* is required for cell viability and perhaps cell cycle progression (287) and it may play a significant role in a number of diverse activities such as DNA replication and transcription. Both CtrA and CcrM transcription are influenced by the state of promoter methylation (288,289)). The DNA methyltransferase, CcrM, methylates the target sequence GANTC at the A residue and is under tight cell cycle control being expressed during the later stages of S-phase when the chromosomes have finished duplicating but the cells have not yet divided. The regulation of CcrM is under transcriptional control by CtrA and proteolytic turnover by Lon protease (242). Proteolysis of CcrM is constitutive and the increased transcription of CcrM in late S-phase saturates Lon activity allowing the protein levels to peak during this period of the cell cycle. This contrasts with *E. coli* Dam which is constitutively expressed and active (9). The *Caulobacter* chromosome, like that of *E. coli,* alternates between states of fully methylated and hemimethylated DNA during DNA replication. At the end of DNA replication, most of the DNA is found in the hemimethylated state with the regions proximal to *Cori* remaining hemimethylated longer (290). Unlike *E. coli*, the pattern of CcrM methylation shows that *Caulobacter* replication initiates only once per cell cycle. In DNA methylation state assays, unmethylated DNA is a hallmark of over-replication. The presence of unmethylated DNA is a typical feature of promiscuous plasmid replication in *Caulobacter* (274,291). However in the chromosomal context unmethylated DNA is difficult to detect (290) suggesting CcrM plays an important role in repressing chromosome replication.

5. Central Theme of this Thesis

This thesis focuses on the negative regulation of chromosome replication in *Caulobacter crescentus* and aims to explain how chromosome replication is restricted to just one round per cell cycle. My studies begin with the CtrA response regulator protein, because CtrA was a proposed negative regulator of chromosome replication in swarmer cells. My thesis extends this hypothesis and addresses whether CtrA also acts to control replication in stalked cells. I therefore studied CtrA binding to the replication origin and I monitored CtrA activity throughout the cell cycle *in vivo* using a well defined chromatin-immunoprecipitation assay. I discovered a new class of CtrA DNA binding sites and I demonstrated that DNA-binding and transcription-regulation are separable *in vivo* activities of CtrA. I developed a quantitative *in vivo* protein-DNA cross-linking assay to monitor CtrA binding to the replication origin. This assay also allowed me to discover that the protein chaperone ClpX contacts the chromosome replication origin precisely at the start of the replication cycle. My results have significantly changed our model of how CtrA can regulate the cell cycle. These results also suggest that a new role for ClpX is to disassemble proteins at the replication origin. I end my thesis by linking negative regulation of chromosome replication in *C. crescentus* with that of *E. coli*. Regulation by CtrA, or similar response regulators is probably not shared between the replication origins of these bacteria. I therefore addressed whether *C. crescentus* also employed an *E. coli*-like "RIDA mechanism" for the "Regulated Inactivation of DnaA." My preliminary work demonstrates that *C. crescentus* has the essential *hda* RIDA

component. I propose that *C. crescentus* likewise employs the RIDA mechanism in cooperation with that of CtrA and ClpX.

FIGURES

Figure 1. Schematic of *E. coli* replication origin *oriC*. Key DNA elements are shown. *oriC* is flanked by two open reading frames, *mioC* and *gidA*. Five filled triangles denote the placement and orientation of five 9-mer DnaA boxes. The arrowheads indicate a series of 13 mer sequences and comprise an AT-rich region which act as unwinding elements. The large open triangle indicates the IHF binding site. Figure adapted from (235).

E. coli oriC

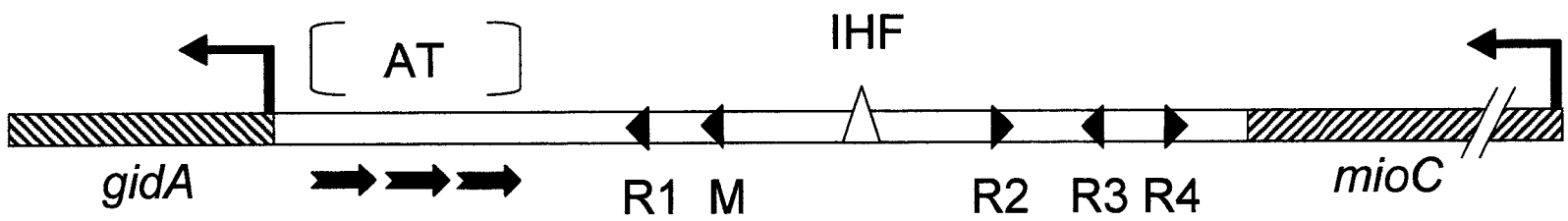


Figure 2. The model of chromosome replication initiation at *oriC*. Five DnaA boxes in *oriC* (9-mers) are targets of DnaA binding. Sequential loading of DnaA at *oriC* forms an oligomeric complex that facilitates the melting and unwinding of the origin DNA (13-mers). The open complex permits the loading of the DnaB by both DnaA and DnaC to form the pre-priming complex. Figure adapted from (17).

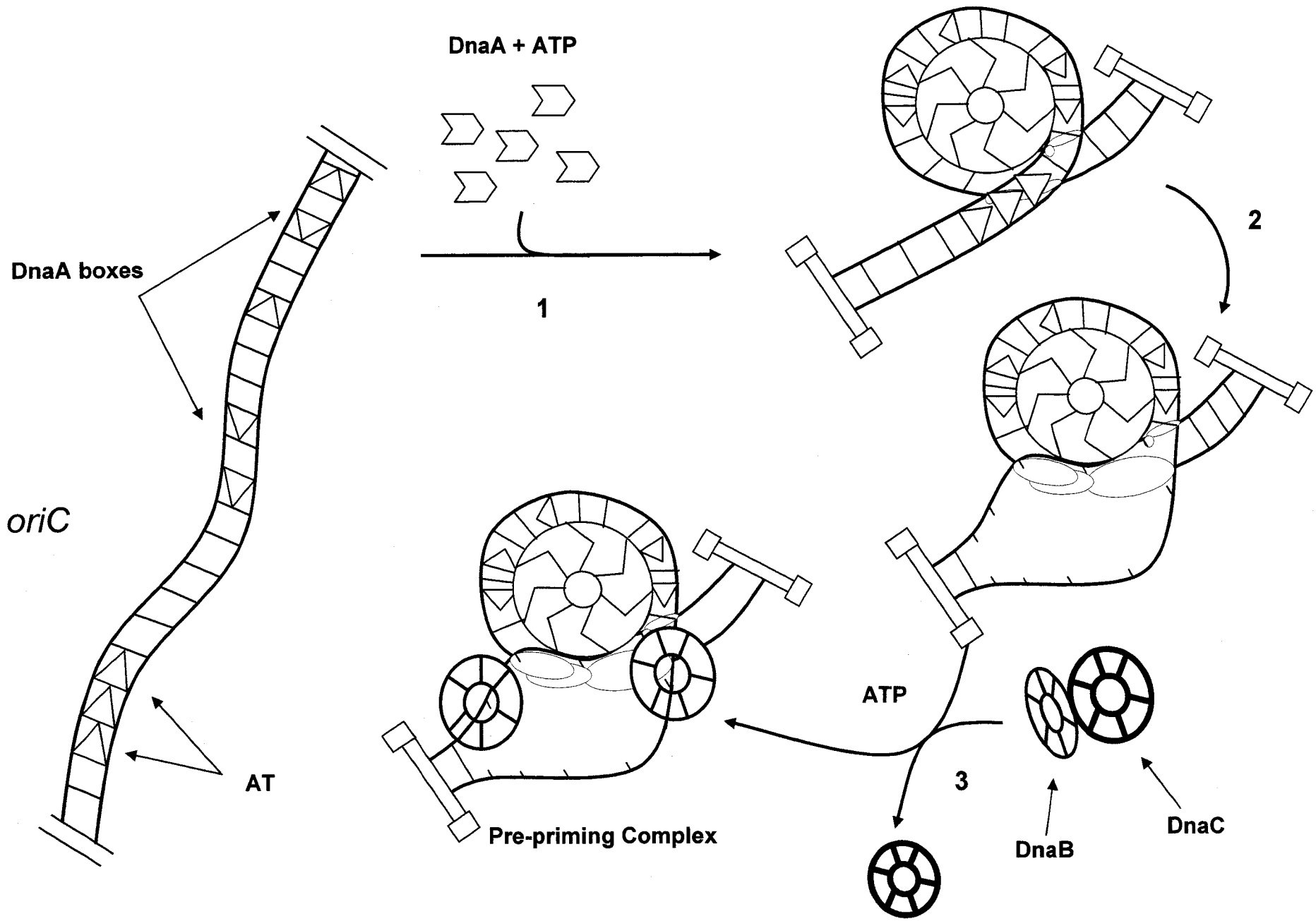

DnaA + ATP
DnaA boxes
oriC
AT
1
2
3
ATP
Pre-priming Complex
DnaB
DnaC

Figure 3. Model of a DNA replication fork. The major components of the DNA replication fork are shown. Two oppositely-oriented DNA polymerase III molecules (green) are tethered to DNA by the DnaN beta-clamp (purple ring). The tau subunit (brown) couples the leading and lagging strand polymerases to form the replication fork. RNA primase (blue triangle) synthesizes RNA primer for both leading and lagging strand synthesis. The DnaB helicase (blue ring) moves ahead of the replication fork and unwinds the parental duplex DNA and single stranded DNA is stabilized by single stranded binding proteins (SSB: yellow). Figure adapted from (17).

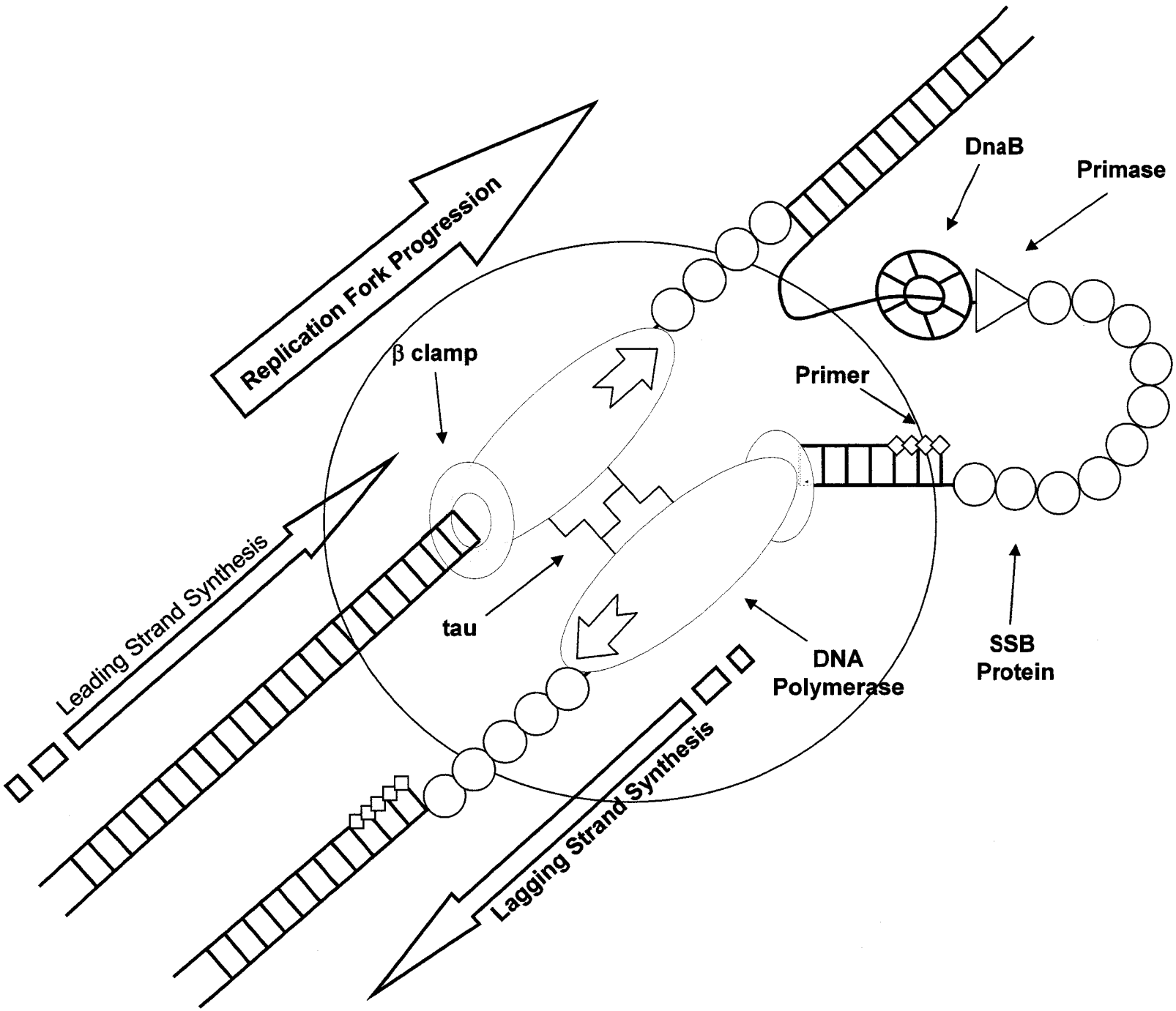

Replication Fork Progression
DnaB
Primase
β clamp
Primer
Leading Strand Synthesis
tau
DNA
Polymerase
SSB
Protein
Lagging Strand Synthesis

Figure 4. Eukaryotic Licensing. The schematic demonstrates the regulated loading of Mcm2-7 proteins on replication origins (triangles) during the yeast replication origin. Two origins of replication are shown. During late mitosis (M), the replication licensing system becomes active and allows Mcm2-7 complexes (red) to load onto replication origins (green). In late G1 the licensing system is shut off through an inhibition by cyclin dependent kinases. During chromosome replication (S), the Mcm2-7 begin to migrate away from the replication origins ahead of the replication forks and dissociate from the DNA during termination. As cells enter G2 and replication completes, Mcm2-7 are not permitted to reassociates with DNA until cell have successfully completed mitosis (adapted from (292)).

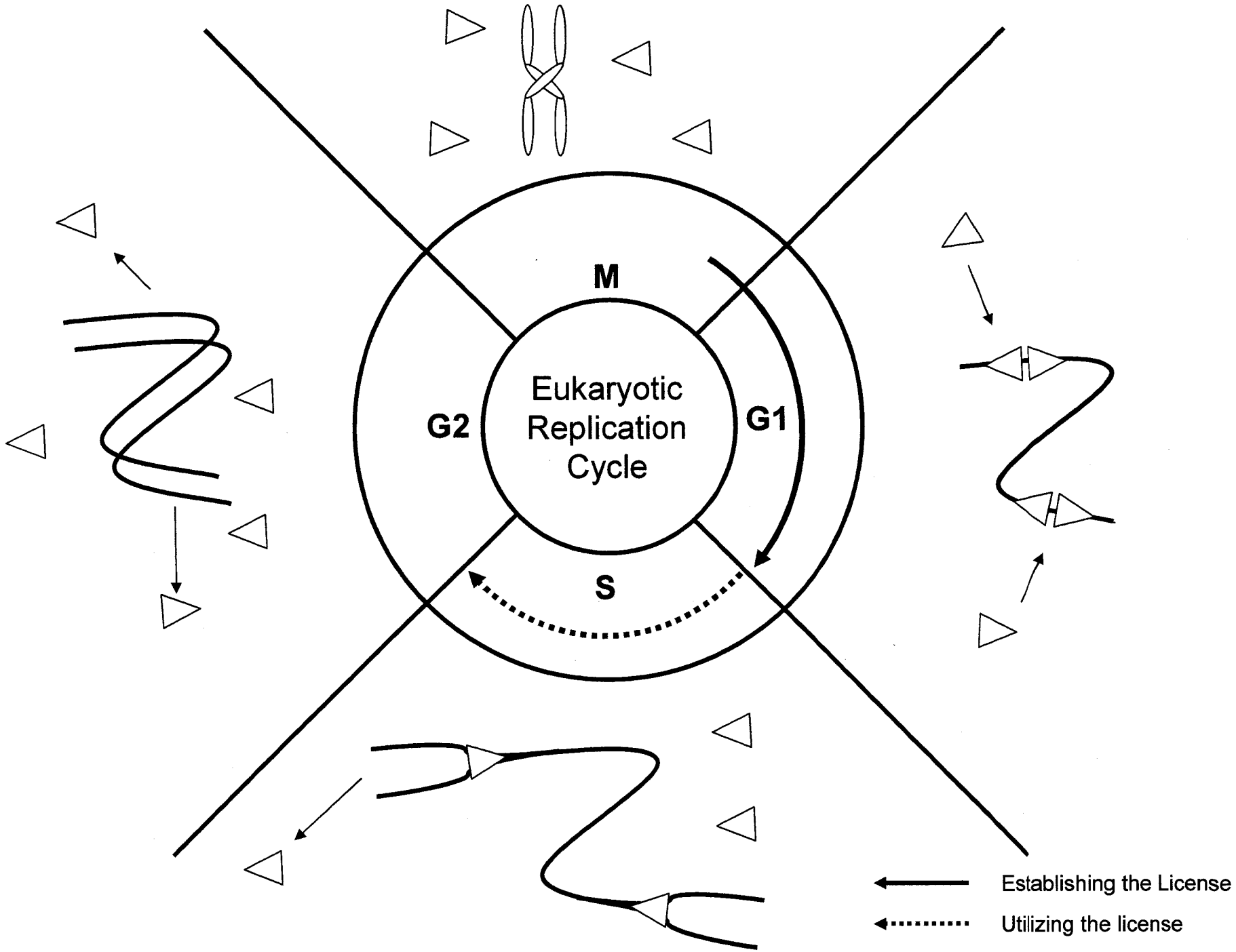

M
G2
Eukaryotic
Replication
Cycle
G1
S
Establishing the License
Utilizing the license

Figure 5. Mechanisms that regulate DnaA activity. Three mechanisms negatively regulate chromosome replication in *E. coli*. 1) DnaN and Hda are components of Regulated Inactivation of DnaA (RIDA) that stimulate the conversion of DnaA-ATP (active) to DnaA-ADP (inactive). 2) The *datA* locus, a region close to *oriC*, is duplicated by the moving replication forks (RF) and acts as a nucleating point for DnaA. datA functions to reduce the availability of DnaA *in vivo* and prevent re-replication. The third mechanism, sequestration, is controlled by the SeqA protein which binds the newly replicated and hemimethylated origin DNA (-CH3), sequestering it at the membrane and blocking reassociation of DnaA.

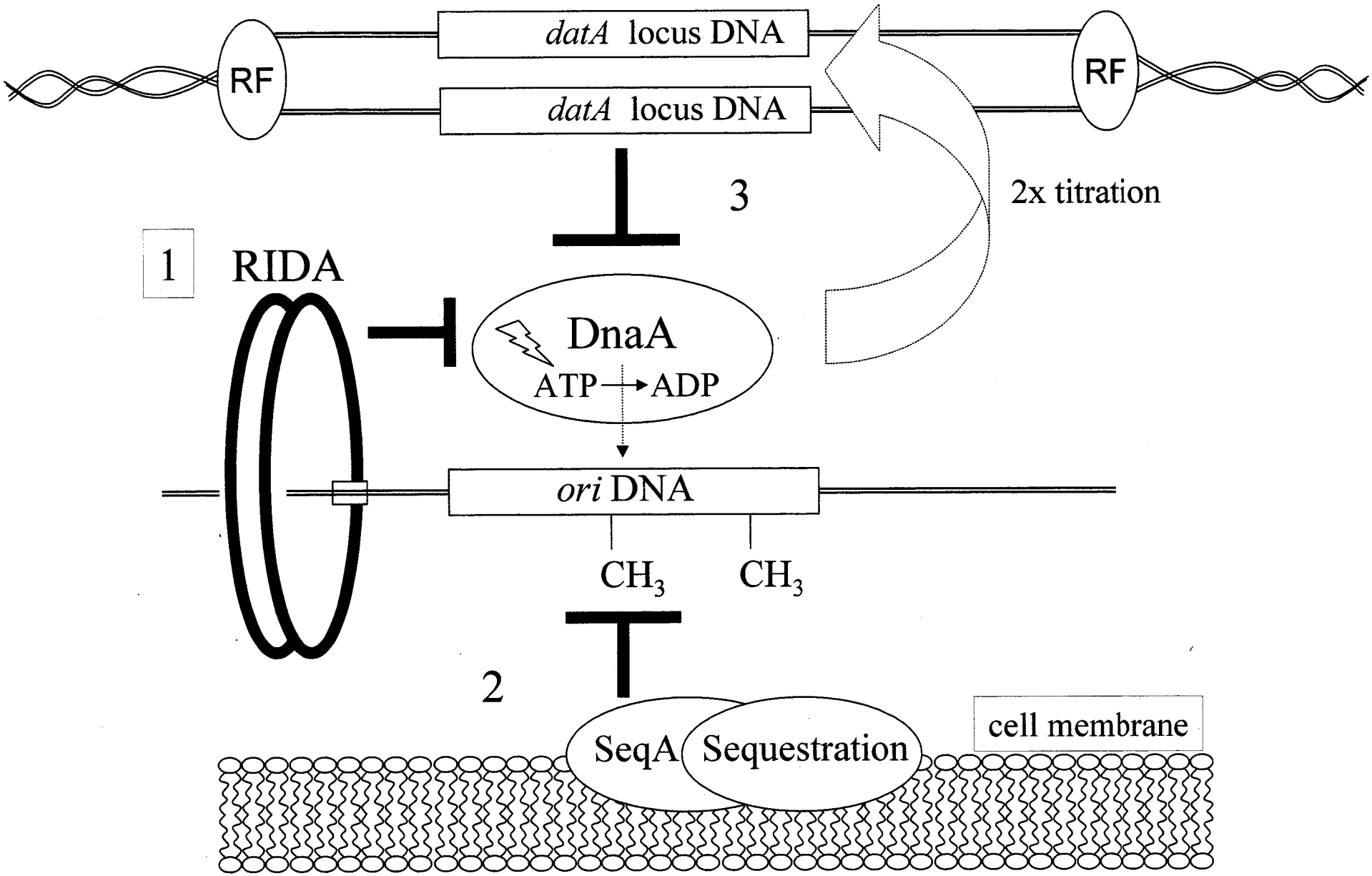

datA locus DNA
datA locus DNA
RF
RF
3
2x titration
1
RIDA
DnaA
ATP → ADP
ori DNA
CH3
CH3
2
SeqA
Sequestration
cell membrane

Figure 6. Schematic of Two Component Systems. Two component systems are comprised of a membrane bound histidine protein kinase (HPK) and a cytoplasmic response regulator. The ATPase domain of the HPK catalyzes the autophosphorylation of a conserved histidine residue (H). The phosphate is subsequently transferred to a conserved aspartate residue (D) on the RR (receiver domain). Phosphorylation of the RR stimulates the effector domain and execution of the response. Figure adapted from (170).

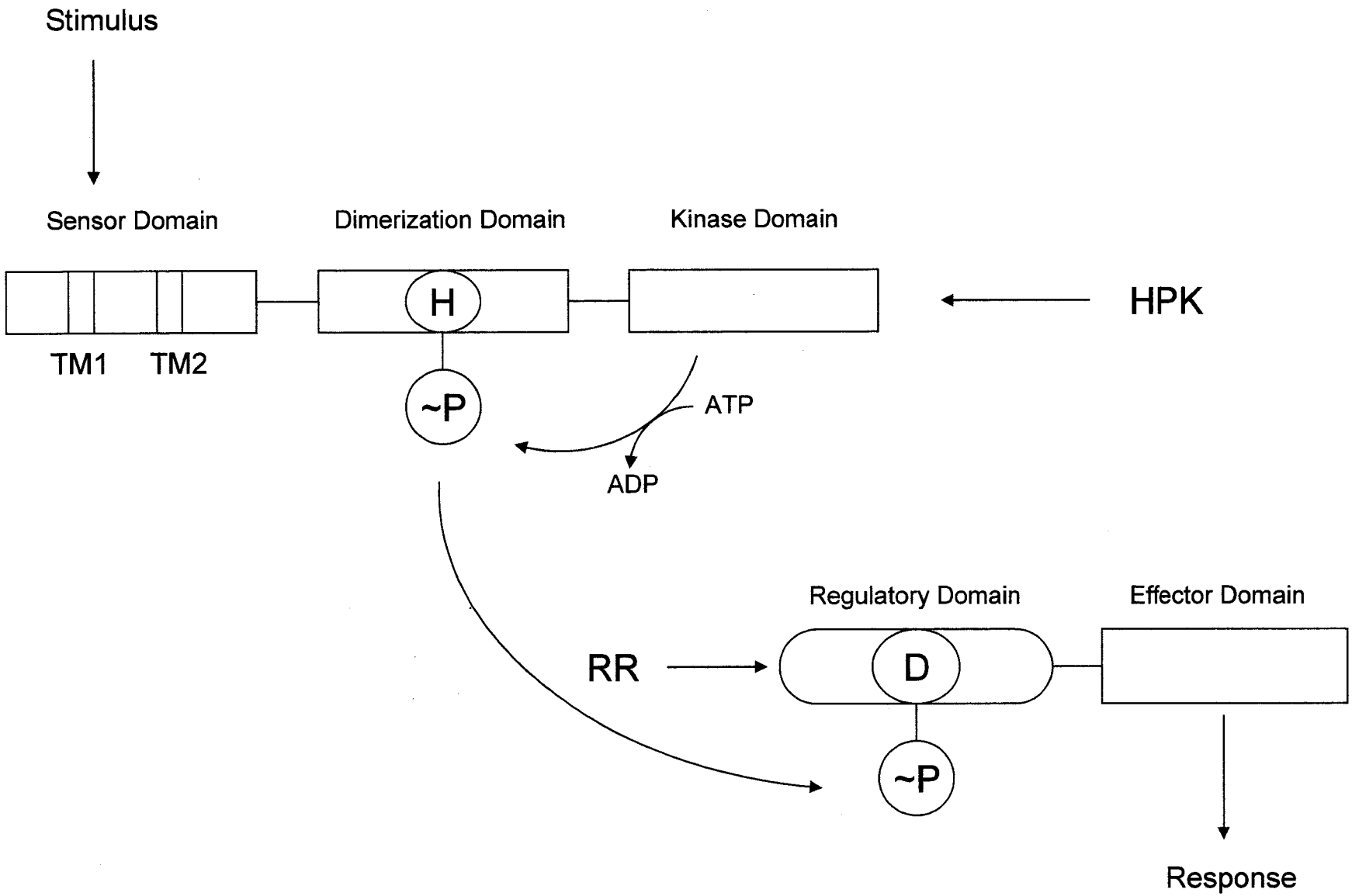

Stimulus
Sensor Domain
Dimerization Domain
Kinase Domain
H
HPK
TM1
TM2
~P
ATP
ADP
Regulatory Domain
Effector Domain
RR
D
~P
Response

Figure 7. The *Caulobacter crescentus* cell cycle. The cycle begins with a non-replicating chemotactic swarmer cell (Sw) which differentiates to the replicating stalk cell (St). Growth of the pre-divisional cell produces a new flagellated swarmer pole. Segregating chromosomes are positioned in both the non-replicating swarmer (rep-) and the replication competent stalk cell (rep+). Shading indicates the temporal and spatial presence of the CtrA response regulator. Figure adapted from (235).

Caulobacter crescentus cell cycle

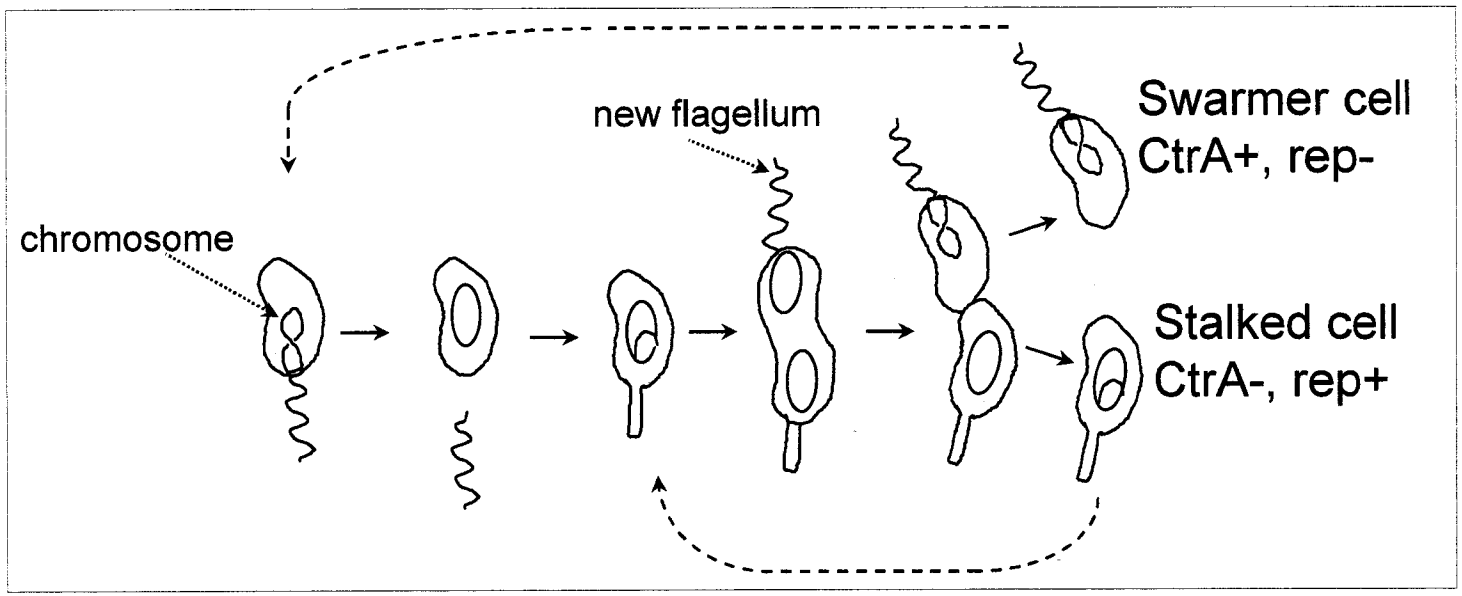
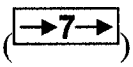
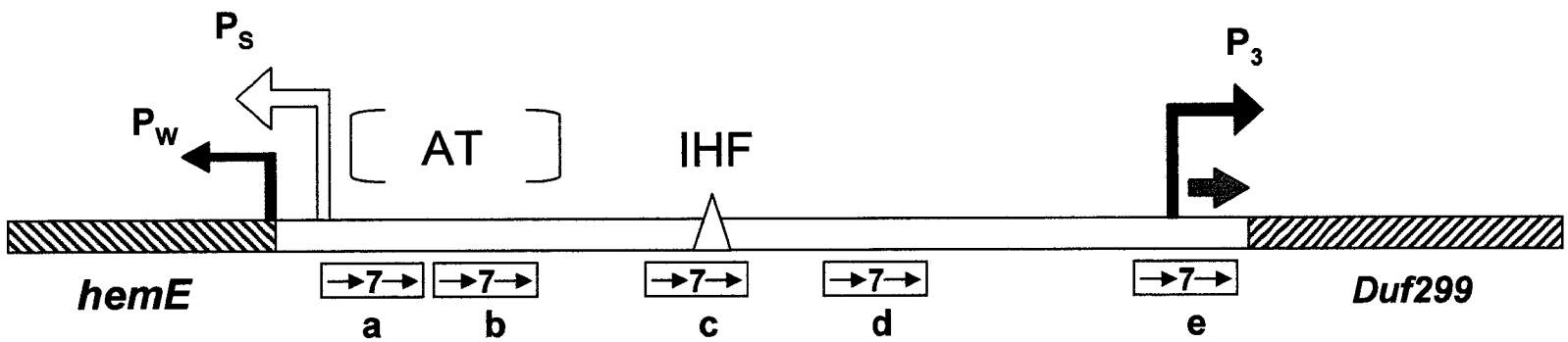


Figure 8. Conspicuous features of the *Caulobacter* Origin of Replication (*Cori*).

Five binding sites (**a-e**) () for the response regulator CtrA are shown including a single IHF overlapping CtrA binding site **c** (open triangle). A highly conserved *E. coli* - like DnaA box is located near CtrA binding site **e** in the rightward half of *Cori* (open arrow). *Cori* is flanked by two open reading frames, *hemE* and *Duf299* (formerly *RP001*) and includes three promoters, the weak promoter (P_W), the strong promoter (P_S) and the P_3 promoter. Also shown is an AT rich sequence where melting of the origin DNA by DnaA is presumed to happen. Figure adapted from (235).

C. crescentus Cori



REFERENCES

1. Jacob, F., Brenner, S. and Cuzin, F. (1963) On the regulation of DNA replication in bacteria. *Cold Spring Harbor Symposia on Quantitative Biology*, **28**, 329-348.
2. Davey, M.J., Jeruzalmi, D., Kuriyan, J. and M, O.D. (2002) Motors and switches: AAA+ machines within the replisome. *Nat Rev Mol Cell Biol*, **3**, 826-835.
3. Oka, A., Sugimoto, K., Takanami, M. and Hirota, Y. (1980) Replication origin of the Escherichia coli K-12 chromosome: the size and structure of the minimum DNA segment carrying the information for autonomous replication. *Mol Gen Genet*, **178**, 9-20.
4. Zyskind, J.W., Cleary, J.M., Brusilow, W.S.A., Harding, N.E. and Smith, D.W. (1983) Chromosome Replication Origin from the Marine Bacterium *Vibrio harveyi* Functions in *Escherichia coli*: *oriC* Consensus Sequence. *Proc. Natl. Acad. Sci. USA*, **80**, 1164-1168.
5. Fuller, R.S., Funnell, B.E. and Kornberg, A. (1984) The DnaA Protein Complex with the *E. coli* Chromosomal replication Origin (*oriC*) and Other DNA SIte. *Cell*, **38**, 889-900.
6. Bramhill, D. and Kornberg, A. (1988) A Model for the Initiation at Origins of Replication. *Cell*, **54**, 915-918.
7. Bremer, H. and Churchward, G. (1991) Control of cyclic chromosome replication in Escherichia coli. *Microbiol Rev*, **55**, 459-475.

8. Bakker, A. and Smith, D.W. (1989) Methylation of GATC Sites is Required for Precise Timing between Rounds of DNA Replication in *Escherichia coli*. *Journal of Bacteriology*, **171**, 5738-5742.

9. Boye, E. and Lobner-Olesen, A. (1990) The Role of *dam* Methyltransferase in the Control of DNA Replication in *E. coli*. *Cell*, **62**, 981-989.

10. Landoulsi, A., Malki, A., Kern, R., Kohiyama, M. and Hughes, P. (1990) The *E. coli* Cell Surface Specifically Prevents the Initiation of DNA Replication at *oriC* on Hemimethylated DNA Templates. *Cell*, **63**, 1053-1060.

11. Ogden, G.B., Pratt, M.J. and Schaechter, M. (1988) The replicative origin of the E. coli chromosome binds to cell membranes only when hemimethylated. *Cell*, **54**, 127-135.

12. Schmid, M.B. (1990) More than just "histone-like" proteins. *Cell*, **63**, 451-453.

13. Grimwade, J.E., Ryan, V.T. and Leonard, A.C. (2000) IHF redistributes bound initiator protein, DnaA, on supercoiled *oriC* of *Escherichia coli*. *Molecular Microbiology*, **35**, 835-844.

14. Ryan, V.T., Grimwade, J.E., Nievera, C.J. and Leonard, A.C. (2002) IHF and HU stimulate assembly of pre-replication complexes at Escherichia coli oriC by two different mechanisms. *Mol Microbiol*, **46**, 113-124.

15. Ryan, V.T., Grimwade, J.E., Camara, J.E., Crooke, E. and Leonard, A.C. (2004) Escherichia coli prereplication complex assembly is regulated by dynamic interplay among Fis, IHF and DnaA. *Mol Microbiol*, **51**, 1347-1359.

16. Fillutowicz, M., Ross, W., Wild, J. and Gourse, R.L. (1992) Involvement of Fis in the replication of the *Escherichia coli* Chromosome. *Journal of Bacteriology*, **174**, 398-407.

17. Baker, T.A. and Wickner, S.H. (1992) Genetics and Enzymology of DNA Replication in *Escherichia coli*. *Annual Reviews in Genetics*, **26**, 447-477.

18. Ogawa, T., Pickett, G.G., Kogoma, T. and Kornberg, A. (1984) RNAase H confers specificity in the *dnaA*-dependent initiation of replication at the unique origin of the *Escherichia coli* chromosome *in vivo* and *in vitro*. *Proc. Natl. Acad. Sci. USA*, **81**, 1040-1044.

19. Torrey, T.A., Atlung, T. and Kogoma, T. (1984) dnaA suppressor (dasF) mutants of Escherichia coli are stable DNA replication (sdrA/rnh) mutants. *Mol. Gen. Genet*, **196**, 350-355.

20. Carr, K.M. and Kaguni, J.M. (2001) Stoichiometry of DnaA and DnaB protein in initiation at the Escherichia coli chromosomal origin. *J Biol Chem*, **276**, 44919-44925.

21. Sekimizu, K., Bramhill, D. and Kornberg, A. (1988) Sequential early stages in the in vitro initiation of replication at the origin of the Escherichia coli chromosome. *J Biol Chem*, **263**, 7124-7130.

22. Sekimizu, K., Bramhill, D. and Kornberg, A. (1987) ATP activates dnaA protein in initiating replication of plasmids bearing the origin of the E. coli chromosome. *Cell*, **50**, 259-265.

23. Bramhill, D. and Kornberg, A. (1988) Duplex Opening by DnaA Protein at Novel Sequences in Initiation of Replication at the Origin of the *E. coli* Chromosome. *Cell*, **52**, 743-755.

24. Yung, B.Y.-M. and Kornberg, A. (1989) The DnaA Initiator Protein Binds Separate Domains in the Replication Origin of *Escherichia coli*. *The Journal of Biological Chemistry*, **264**, 6146-6150.

25. Skarstad, K., Baker, T.A. and Kornberg, A. (1990) Strand Separation Required for Initiation of Replication at the Chromosome Origin of *E. coli* is Facilitated by a Distant RNA-DNA Hybrid. *The EMBO Journal*, **9**, 2341-2348.

26. Funnell, B.E., Baker, T.A. and Kornberg, A. (1987) In vitro assembly of a prepriming complex at the origin of the Escherichia coli chromosome. *J Biol Chem*, **262**, 10327-10334.

27. Wahle, E., Lasken, R.S. and Kornberg, A. (1989) The DnaB-DnaC replication protein complex of *Escherichia coli*. II. Role of the complex in mobilizing DnaB functions. *Journal of Biological Chemistry*, **264**, 2469-2475.

28. Masai, H., Nomura, N. and Arai, K. (1990) The ABC-primosome. A novel priming system employing DnaA, DnaB, DnaC, and primase on a hairpin containing a DnaA box sequence. *Journal of Biological Chemistry*, **265**, 1-33.

29. Wickner, S. and Hurwitz, J. (1975) Interaction of *Escherichia coli* DnaB and DnaC (D) gene products in vitro. *Proceedings of the National Academy of Sciences. USA*, **72**, 921-925.

30. Kobori, J.A. and Kornberg, A. (1982) The Escherichia coli DnaC gene product. II. Purification, physical properties, and role in replication. *Journal of Biological Chemistry*, **257**, 13763-13769.

31. Wahle, E., Lasken, R.S. and Kornberg, A. (1989) The DnaB-DnaC replication protein complex of *Escherichia coli*. I. Formation and properties. *Journal of Biological Chemistry*, **264**, 2463-2468.

32. Allen, G.C.J. and Kornberg, A. (1991) Fine balance in the regulation of DnaB helicase by DnaC protein in replication in *Escherichia coli*. *Journal of Biological Chemistry*, **266**, 22096-22101.

33. Baker, T.A., Funnell, B.E. and Kornberg, A. (1987) Helicase action of DnaB protein during replication from the Escherichia coli chromosomal origin in vitro. *Journal of Biological Chemistry*, **262**, 6877-6885.

34. Baker, T.A. and Kornberg, A. (1988) Transcription Activation of Initiation of Replication from the *E. coli* Chromosomal Origin: An RNA-DNA Hybrid Near *oriC*. *Cell*, **55**, 113-123.

35. Kaguni, J.M., Fuller, R.S. and Kornberg, A. (1982) Enzymatic replication of E. coli chromosomal origin is bidirectional. *Nature*, **296**, 623-627.

36. Asai, T., Chen, C.-P., Nagata, T., Takanami, M. and Imai, M. (1992) Transcription *In Vivo* within the Replication Origin of the *Escherichia coli* Chromosome: A Mechanism for Activating Initiation of Replication. *Mol Gen Genet*, **231**, 169-178.

37. Asai, T., Takanami, M. and Imai, M. (1990) The AT Richness and gid Transcription Determine the Left Border of the Replication Origin of the *E. coli* Chromosome. *The EMBO Journal*, **9**, 4065-4072.

38. Ogawa, T. and Okazaki, T. (1991) Concurrent transcription from the gid and mioC promoters activates replication of an Escherichia coli minichromosome. *Mol Gen Genet*, **230**, 193-200.

39. Lohman, T.M. and Bjornson, K.P. (1996) Mechanisms of helicase-catalyzed DNA unwinding. *Annu Rev Biochem*, **65**, 169-214.

40. Baker, T.A., Sekimizu, K., Funnell, B.E. and Kornberg, A. (1986) Extensive unwinding of the plasmid template during staged enzymatic initiation of DNA replication from the origin of the Escherichia coli chromosome. *Cell*, **45**, 53-64.

41. LeBowitz, J.H. and McMacken, R. (1986) The Escherichia coli DnaB replication protein is a DNA helicase. *Journal of Biological Chemistry*, **261**, 4738-4748.

42. Wu, C.A., Zechner, E.L. and Marians, K.J. (1992) Coordinated leading- and lagging-strand synthesis at the Escherichia coli DNA replication fork. I. Multiple effectors act to modulate Okazaki fragment size. *Journal of Biological Chemistry*, **267**, 4074-4083.

43. Bouche, J.P., Rowen, L. and Kornberg, A. (1978) The RNA primer synthesized by primase to initiate phage G4. *Journal of Biological Chemistry*, **253**, 765-769.

44. Arai, K., Low, R., Kobori, J., Shlomai, J. and Kornberg, A. (1981) Mechanism of DnaB protein action. V. Association of DnaB proein, protein n' and other prepriming proteins in the primosome of DNA replication. *Journal of Biological Chemistry*, **256**, 5273-5280.

45. Maki, H. and Kornberg, A. (1985) The polymerase subunit of DNA polymerase III of *Escherichia coli. Journal of Biological Chemistry*, **263**, 15008-15015.

46. Sheuermann, R.H. and Echols, H. (1985) A separate editing exonuclease for DNA replication: the epsilon subunit of *Escherichia coli* DNA polymerase III holoenzyme. *Proceedings of the National Academy of Sciences. USA*, **81**, 7747-7751.

47. DeRose, E.F., Darden, T., Harvey, S., Gabel, S., Perrino, F.W., Schaaper, R.M. and London, R.E. (2003) Elucidation of the epsilon-theta subunit interface of Escherichia coli DNA polymerase III by NMR spectroscopy. *Biochemistry*, **42**, 3635-3644.

48. Maki, S. and Kornberg, A. (1988) DNA polymerase III holoenzyme of *Escherichia coli.* I. Purification and distinctive functions of subunits tau and gamma, the *dnaZX* gene products. *The Journal of Biological Chemistry*, **263**.

49. Maki, S. and Kornberg, A. (1988) DNA polymerase III holoenzyme of Escherichia coli. III. Distinctive processive polymerases reconstituted from purified subunits. *J Biol Chem*, **263**, 6561-6569.

50. McHenry, C.S. (1982) Purification and characterization of DNA polymerase III. Identification of tau as a subunit of the DNA polymerase III holoenzyme. *Journal of Biological Chemistry*, **257**, 2657-2663.

51. Studwell-Vaughan, P.S. and O'Donnell, M. (1991) Constitution of the twin polymerase of DNA polymerase III holoenzyme. *Journal of Biological Chemistry*, **266**, 19833-19841.

52. Xiao, H., Naktinis, V. and M, O.D. (1995) Assembly of a chromosomal replication machine: two DNA polymerases, a clamp loader, and sliding clamps in one holoenzyme particle. IV. ATP-binding site mutants identify the clamp loader. *J Biol Chem*, **270**, 13378-13383.

53. Kong, X.P., Onrust, R., M, O.D. and Kuriyan, J. (1992) Three-dimensional structure of the beta subunit of E. coli DNA polymerase III holoenzyme: a sliding DNA clamp. *Cell*, **69**, 425-437.

54. Stukenberg, P.T., Studwell-Vaughan, P.S. and O'Donnell, M. (1991) Mechanism of the sliding beta-clamp of DNA polymerase III holoenzyme. *Journal of Biological Chemistry*, **266**, 11328-11334.

55. Turner, J., Hingorani, M.M., Kelman, Z. and O'Donnell, M. (1999) The internal workings of a DNA polymerase clamp-loading machine. *EMBO Journal*, **18**, 771-783.

56. Stewart, J., Hingorani, M.M., Kelman, Z. and O'Donnell, M. (2001) Mechanism of beta-clamp opening by the delta-subunit of Escherichia coli DNA polymerase III holoenzyme. *Journal of Biological Chemistry*, **276**, 19182-19189.

57. Naktinis, V., Onrust, R., Fang, L. and M, O.D. (1995) Assembly of a chromosomal replication machine: two DNA polymerases, a clamp loader, and sliding clamps in one holoenzyme particle. II. Intermediate complex between the clamp loader and its clamp. *J Biol Chem*, **270**, 13358-13365.

58. Jeruzalmi, D. (2001) Mechanism of processivity clamp opening by the delat-subunit wrench of the clamp loader complex of E. coli DNA polymerase III. *Cell*, **106**, 417-428.

59. Jeruzalmi, D., O'Donnell, M. and Kuriyan, J. (2001) Crystal stucture of the processivity clamp loader gamma complex of E. coli DNA polymerase III. *Cell*, **106**, 429-441.

60. Yuzhakov, A., Kelman, Z. and O'Donnell, M. (1999) Trading Places on DNA- A Three-Point Switch Underlies Primer Handoff from Primase to the Replicative DNA Polymerase. *Cell*, **96**, 153-163.

61. Xiao, H., Dong, Z. and O'Donnell, M. (1993) DNA Polymerase III accessory proteins. IV. Characterization of chi and psi. *Journal of Biological Chemistry*, **268**, 11779-117784.

62. Maki, S. and Kornberg, A. (1988) DNA polymerase III holoenzyme of *Escherichia coli*. II. A novel complex including the gamma subunit essential for processive synthesis. *Journal of Biological Chemistry*, **263**, 6555-6560.

63. Onrust, R., Stukenberg, P.T. and O'Donnell, M. (1991) Analysis of the ATPase subassembly which initiates processive DNA synthesis by DNA polymerase III holoenzyme. *Journal of Biological Chemistry*, **266**, 21681-21686.

64. Funnell, B.E., Baker, T.A. and Kornberg, A. (1986) Complete enzymatic replication of plasmids containing the origin of the Escherichia coli chromosome. *Journal of Biological Chemistry*, **261**, 5616-5624.

65. Wang, J.C. (1985) DNA topoisomerases. *Annual Review of Biochemistry*, **54**, 665-697.

66. Wu, C.A., Zechner, E.L., Reems, J.A., McHenry, C.S. and Marians, K.J. (1992) Coordinated leading- and lagging-strand synthesis at the Escherichia coli DNA

replication fork. V. Primase action regulates the cycle of Okazaki fragment synthesis. *Jouranl of Biological Chemistry*, **267**, 4074-4083.

67. Zechner, E.L., Wu, C.A. and Marians, K.J. (1992) Coordinated leading and lagging strand synthesis at the *Escherichia coli* DNA replication fork. III. A polymerase-primase interaction governs primer size. *Journal of Biological Chemistry*, **267**, 4054-4063.

68. Zechner, E.L., Wu, C.A. and Marians, K.J. (1992) Coordinated leading and lagging strand synthesis at the *Escherichia coli* DNA replication fork. II. Frequency of primer synthesis and efficiency of primer utilization control Okazaki fragment size. *Journal of Biological Chemistry*, **267**, 4045-4053.

69. Hill, T.M., Kopp, B.J. and Kuempel, P.L. (1988) Termination of DNA replication in Escherichia coli requires a trans-acting factor. *J Bacteriol*, **170**, 662-668.

70. Pelletier, A.J., Hill, T.M. and Kuempel, P.L. (1988) Location of sites that inhibit progression of replication forks in the terminus region of Escherichia coli. *J Bacteriol*, **170**, 4293-4298.

71. Hidaka, M., Kobayashi, T., Takenaka, S., Takeya, H. and Horiuchi, T. (1989) Purification of the DNA replication terminus (ter) site-binding protien in *Escherichia coli* and identification of the structural gene. *Journal of Biological Chemistry*, **264**, 21031-21037.

72. Khatri, G.S., MacAllister, T., Sista, P.R. and Bastia, D. (1989) The replication terminator protein of E. coli is a DNA sequence-specific contra-helicase. *Cell*, **59**, 667-674.

73. Lee, E.H., Kornberg, A., Hidaka, M., Kobayashi, T. and Horiuchi, T. (1989) *Escherichia coli* replication termination protein impedes the action of helicases. *Proc. Natl. Acad. Sci. USA*, **865**, 9104–9108.

74. Kamada, K., Horiuchi, T., Ohsumi, K., Shimamoto, N. and Morikawa, K. (1996) Structure of a replication-terminator protein complexed with DNA. *Nature*, **383**, 598-603.

75. Neylon, C., Brown, S.E., Kralicek, A.V., Miles, C.S., Love, C.A. and Dixon, N.E. (2000) Interaction of the *Escherichia coli* replication terminator protein (Tus) with DNA: a model derived from DNA-binding studies of mutant proteins by surface plasmon resonance. **39**, 11989–11999.

76. Henderson, T.A., Nilles, A.F., Valjavec-Gratian, M. and T.M., H. (2001) Site-directed mutagenesis and phylogenetic comparisons of the *Escherichia coli* Tus protein: DNA-protein interactions alone can not account for Tus activity. *Mol. Genet. Genomics*, **265**, 941–953.

77. Adams, D.E., Shekhtman, E.M., Zechiedrich, E.L., Schmid, M.B. and Cozzarelli, N.R. (1992) The role of topoisomerase IV in partitioning bacterial replicaons and the structure of catenated intermediates in DNA replication. *Cell*, **71**, 277-288.

78. Espeli, O., Lee, C. and Marians, K.J. (2003) A physical and functional interaction between Escherichia coli FtsK and topoisomerase IV. *J Biol Chem*, **278**, 44639-44644.

79. Steiner, W.W. and Kuempel, P.L. (1998) Cell division is required for resolution of dimer chromosomes at the dif locus of Escherichia coli. *Mol Microbiol*, **27**, 257-268.

80. Blakely, G., Colloms, S., May, G., Burke, M. and Sherratt, D. (1991) Escherichia coli XerC recombinase is required for chromosomal segregation at cell division. *New Biol*, **3**, 789-798.

81. Steiner, W., Liu, G., Donachie, W.D. and Kuempel, P. (1999) The cytoplasmic domain of FtsK protein is required for resolution of chromosome dimers. *Mol Microbiol*, **31**, 579-583.

82. Marahrens, Y. and Stillman, B. (1992) A Yeast Chromosome Origin of DNA Replication Defined by Multiple Functional Elements. *Science*, **255**, 817-823.

83. Newlon, C.S. (1996) In DePamphilis, M. L. (ed.), *DNA replciation in Eukaryotic cells*. Cold Spring Harbor Lab. Press, Plainview, NY, pp. 873-914.

84. Van Houten, J.V. and Newlon, C.S. (1990) Mutational analysis of the consensus sequence of a replication origin from yeast chromosome III. *Mol Cell Biol*, **10**, 3917-3925.

85. Neuwald, A.F., Aravind, L., Spouge, J.L. and Koonin, E.V. (1999) AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Research*, **9**, 27-43.

86. Rao, H. and Stillman, B. (1995) *Proc. Natl. Acad. Scie. USA*, **92**, 2224-2228.

87. Rao, H., Marahrens, Y. and Stillman, B. (1994) *Mol. Cell. Biol.*, **14**, 7643-7651.

88. Diffley, J.F.X. and Stillman, B. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2120-2124.

89. Mechali, M. (2001) DNA replication orgins: from sequence specificity to epigenetics. *Nature Reviews Genetics*, **2**, 640-645.

90. Newton, C.S. and Theis, J.F. (1993) The structure and function of yeast ARS elements. *Curr. Opin. Genet. Dev.*, **3**, 752-758.

91. Linskens, M. and Huberman, J.A. (1988) Organization of replication in ribosomal DNA in *Saccharomyces cerevisiae. Molecular and Cellular Biology*, **8**, 4927-4935.

92. Friedman, K.L., Brewer, B.J. and Fangman, W.L. (1997) Replication profile of Saccharomyces cerevisiae chromosome VI. *Genes to Cells*, **2**.

93. Blow, J.J. and Dutta, A. (2005) Preventing re-replication of chromosomal DNA. *Nat Rev Mol Cell Biol*, **6**, 476-486.

94. Bell, S.P. and Stillman, B. (1992) ATP-dependent Recognition of Eukaryotic Origins of DNA Replication by a Multiprotein Complex. *Nature*, **357**, 128-134.

95. Dutta, A. and Bell, S.P. (1997) *Annu. Rev. Cell. Dev. Biol.*, **13**, 293-332.

96. Lee, D.G. and Bell, S.P. (1997) Architecture of the yeast origin recognition complex bound to origins of DNA replication. *Molecular and Cellular Biology*, **17**, 7159-7168.

97. Klemm, R.D., Austin, R.J. and Bell, S.P. (1997) Coordinate binding of ATP and origin DNA regulates the ATPase activity of origin recognition complex. *Cell*, **88**, 493-502.

98. Aparicio, O.M., Weinstein, D.M. and Bell, S.P. (1997) Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase. *Cell*, **91**, 59-69.

99. Tanaka, T., Knapp, D. and Nasmyth, K. (1997) Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. *Cell*, **90**, 649-660.

100. Diffley, J.F.X., Cocker, J.H., Dowell, S.J. and Rowley, A. (1994) *Cell*, **78**, 303-316.

101. Fox, C.A., Loo, S., Dillin, A. and Rine, J. (1995) The Origin Recognition Complex has Essential Functions in Transcriptional Silencing and Chromosomal Replication. *Genes and Development*, **9**, 911-924.

102. Liang, C., Weinreich, M. and Stillman, B. (1995) *Cell*, **81**, 667-676.

103. Piatti, S., Lengauer, C. and Nasmyth, K. (1995) *EMBO Journal*, **14**.

104. Elsasser, S., Lour, F., Wang, B., Campbell, J.L. and Jong, A. (1996) *Mol. Biol. Cell*, **7**, 1723-1735.

105. Greenwood, E., Nishitani, H. and Nurse, P. (1998) *Journal of Cell Science*, **111**, 3101-3108.

106. Ishimi, Y. (1997) *Journal of Biological Chemistry*, **272**, 24508-24513.

107. Kornberg, A. and Baker, T.A. (1992) *DNA Replication*. Freeman, New York.

108. Tye, B.K. (1999) MCM proteins in DNA replication. *Annual Review of Biochemistry*, **68**, 649-686.

109. Hennessy, K.M., Clark, C.D. and Botstein, D. (1990) *Genes and Development*, **4**, 2252-2263.

110. You, Y., Komamura, Y. and Ishimi, Y. (1999) *Mol. Cell. Biol.*, **19**, 8003-8015.

111. Ishimi, Y., Komamura, Y., You, Z. and Kimura, H. (1998) *Journal of Biological Chemistry*, **273**, 8369-8375.

112. Hua, X.H. and Newport, J. (1998) *Journal of Cell Biology*, **140**, 271-281.

113. Rowles, A., Tada, S. and J.J., B. (1999) *Journal of Cell Science*, **112**, 2011-2018.

114. Lee, J.K., Yeo, Y.S. and Hurwitz, J. (2003) *Proc. Natl. Acad. Sci. USA*, **100**, 2334-2339.

115. Homesley, L., Lei, M., Kawasaki, Y., Sawyer, S., Christensen, T. and Tye, B.K. (2000) *Genes and Development*, **14**, 913-926.

116. Merchant, A.M., Kawasaki, Y., Chen, Y., Lei, M. and Tye, B.K. (1997) *Mol. Cell. Biol.*, **17**, 3261-3271.

117. Sawyer, S.L., Cheng, I.H., Chai, W. and Tye, B.K. (2004) *J. Mol. Biol.*, **340**, 195-202.

118. Wohlschlegel, J.A., Dhar, S.K., Prrokhorova, T.A., Dutta, A. and Walter, J.C. (2002) *Mol. Cells*, **9**, 233-240.

119. Zou, L. and Stillman, B. (1998) Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading f Cdc45p onto chromatin. *Science*, **280**, 593-596.

120. Aparicio, O.M., Stout, A.M. and Bell, S.P. (1999) Differential assembly of Cdc45p and DNA polymerase at early and late origins of DNA replication. *Proc. Natl. Acad. Sci. USA*, **96**, 9130-9135.

121. Uchiyama, M., Griffiths, D., Arai, K. and Masai, H. (2001) *Journal of Biological Chemistry*, **276**, 26189-26196.

122. Kukimoto, I., Igaki, H. and Kanda, T. (1999) *European Journal of Biochemistry*, **265**.

123. Kim, S., Dallmann, H.G., McHenry, C.S. and Marians, K.J. (1996) tau couples the leading- and lagging-strand polymerases at the Escherichia coli Dna replication fork. *J Biol Chem*, **271**, 21406-21412.

124. Miyachi, K., Fritzler, M.J. and Tan, E.M. (1978.) *Journal of Immunology*, **121**, 2228–2234.

125. Krishna, T.S., Kong, X.P., Gary, S., Burgers, P.M. and Kuriyan, J. (1994) *Cell*, **79**, 1233-1243.

126. Cullmann, G., Fien, K., Kobayashi, R. and Stillman, B. (1995) *Molecular and Cellular Biology*, **15**, 4661-4671.

127. O'Donnell, M., Onrust, R., Dean, F.B., Chen, M. and Hurwitz, J. (1993) *Nucleic Acids Research*, **21**, 1-3.

128. Wold, M.S. (1997) *Annual Review of Biochemistry*, **66**, 61-92.

129. Conaway, R.C. and Lehman, I.R. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 4585-4588.

130. Conaway, R.C. and Lehman, I.R. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2523-2527.

131. Mozzherin, D.J., McConnell, M., Jasko, M.V., Krayevsky, A.A. and Tan, C.K. (1996) *Journal of Biological Chemistry*, **271**, 31711-31717.

132. Hashimoto, K., Shimizu, K., Nakashima, N. and Sugino, A. (2003) *Biochemistry*, **42**, 1420-14213.

133. Navas, T.A., Sanchez, Y. and Elledge, S.J. (1996) *Genes and Development*, **10**, 2632-2643.

134. Navas, T.A., Zhou, Z. and Elledge, S.J. (1995) *Cell*, **80**, 29-39.

135. Santamaria, D., Viguera, E., Martinez-Robles, M.L., Hyrien, O., Hernandez, P., Krimer, D.B. and Swartzman, J.B. (2000) Bidirectional replication and random termination. *Nucleic Acids Research*, **28**, 2099-2107.

136. Zhu, J., Newlon, C.S. and Huberman, J.A. (1992) Localization of a DNA replication origin and termination zone on chromosome III of *Saccharomyces cerevisiae. Molecular and Cellular Biology*, **12**, 4733-4741.

137. Linskens, M.H. and Huberman, J.A. (1988) Organization of replication of ribosomal DNA in *Saccharomyces cerevisiae. Melecular Cell Biology*, **8**, 4927-4935.

138. Brewer, B.J. and Fangman, W.L. (1988) A replication fork barrier at the 3' end of yeast ribosomal RNA genes. *Cell*, **55**, 637-643.

139. Codlin, S. and Dalgaard, J.Z. (2003) Complex mechanism of site-specific DNA replication termiantion in fission yeast. *EMBO J.*, **22**, 3431-3440.

140. Arcangioli, B. and de Lahondes, R. (2000) Fission yeast switches mating type by a replication-recombination coupled process. *EMBO J.*, **19**, 1389-1396.

141. Arcangioli, B. (1998) A site- and strand-specific DNA break confers asymmetric switching potential in fission yeast. *EMBO J.*, **17**, 4503-4510.

142. Boulikas, T. (1996) Common structural features of replication origins in all life forms. *Journal of Cellular Biochemistry*, **60**, 297-316.

143. Kelman, L.M. and Kelman, Z. (2003) Archaea: an archetype for replication initiation studies? *Mol Microbiol*, **48**, 605-615.

144. Maisnier-Patin, S., Malandrin, L., Birkeland, N.K. and Bernander, R. (2002) Chromosome replication patternsin the hyperthermophilic euryarchaea Archaeoglobus fulgidus and Methanocaldococcus (Methanococcus) jannaschii. *Mol Microbiol*, **45**, 1443-1450.

145. Kennedy, S.P., Ng, W.V., Salzberg, S.L., Hood, L. and DasSarma, S. (2001) Understanding the adaptation of *Halobacterium* species NRC-1 to its extreme environment through computational analysis of its genome sequence. *Genome Research*, **11**, 1641-1650.

146. Grabowski, B. and Kelman, Z. (2003) Archael DNA replication: Eukaryal proteins in a bacterial context. *Annual Review of Biochemistry*, **57**, 487-516.

147. Messer, W. and Noyer-Weidner, M. (1988) Timing and Targeting: The Biological Functions of Dam Methylation in *E. coli*. *Cell*, **54**, 735-737.

148. Zyskind, J.W. and Smith, D.W. (1992) DNA Replication, the Bacterial Cell Cycle, and Cell Growth. *Cell*, **69**, 5-8.

149. Campbell, J.L. and Kleckner, N. (1990) *E. coli oriC* and the *dnaA* Gene Promoter are Sequestered from *dam* Methyltransferase Following the Passage of the Chromosome Replication Fork. *Cell*, **62**, 967-979.

150. Russell, D.W. and Zinder, N.D. (1987) Hemimethylation Prevents DNA Replication in *E. coli*. *Cell*, **50**, 1071-1079.

151. Landousli, A., Hughes, P., Kern, R. and Kohiyama, M. (1989) Dam methuylation and the initiation of DNA replication on oriC polasmids. *Mol. Gen. Genet.*, **216**, 217-223.

152. Boye, E. (1991) The Hemimethylated Origin of *Escherichia coli* can be Initiated *In Vitro*. *Journal of Bacteriology*, **173**, 4537-4539.

153. Lu, M., Campbell, J.L., Boye, E. and Kleckner, N. (1994) SeqA: A Negative Modulator of Replication Initiation in *E. coli*. *Cell*, 77, 413-426.

154. von Freiesleben, U., Rasmussen, K.V. and Schaechter, M. (1994) SeqA Limits DnaA Activity in Replication from *oriC* in *Escherichia coli*. *Molecular Microbiology*, **14**, 763-772.

155. Skarstad, K., Lueder, G., Lurz, R., Speck, C. and Messer, W. (2000) The *Escherichia coli* SeqA protein binds specifically and co-operatively to two sites in hemimethylated and fully methylated *oriC*. *Molecular Microbiology*, **36**, 1319-1326.

156. Slater, S., Wold, S., Lu, M., Boye, E., Skarstad, K. and Kleckner, N. (1995) *E. coli* SeqA protein Binds *oriC* in Two Different Methyl-Modulated Reactions Appropriate to Its Roles in DNA Replication Initiation and Origin Sequestration. *Cell*, **82**, 927-936.

157. Torheim, N.K. and Skarstad, K. (1999) Escherichia coli SeqA protein affects DNA topology and inhibits open complex formation at oriC. *Embo J*, **18**, 4882-4888.

158. Kitagawa, R., Mitsuki, H., Okazaki, T. and Ogawa, T. (1996) A novel DnaA protein-binding site at 94.7 min on the Escherichia coli chromosome. *Mol Microbiol*, **19**, 1137-1147.

159. Kitagawa, R., Ozaki, T., Moriya, S. and Ogawa, T. (1998) Negative control of replication initiation by a novel chromosomal locus exhibiting exceptional affinity for *Escherichia coli* DnaA protein. *Genes and Development*, **12**, 3032-3043.

160. Camara, J.E., Breier, A.M., Brendler, T., Austin, S., Cozzarelli, N.R. and Crooke, E. (2005) Hda inactivation of DnaA is the predominant mechanism preventing hyperinitiation of *Escherichia coli* DNA replication. *EMBO Reports*, **6**, 736-741.

161. Katayama, T., Kubota, T., Kurokawa, K., Crooke, E. and Sekimizu, K. (1998) The Initiator Function of DnaA Protein Is Negatively Regulated by the Sliding Clamp of the *E. coli* Chromosome Replicase. *Cell*, **94**, 61-71.

162. Katayama, T. and Crooke, E. (1995) DnaA protein is sensitive to a soluble factor and is specifically inactivated for initiation of in vitro replication of the Escherichia coli minichromosome. *Journal of Biological Chemistry*, **270**, 9265-9271.

163. Su'etsugu, M., Kawakami, H., Kurokawa, K., Kubota, T., Takata, M. and Katayama, T. (2001) DNA replication-coupled inactivation of DnaA protein *in vitro*: a role for DnaA arginine-334 of the AAA+ box VIII motif in ATP hydrolysis. *Molecular Microbiology*, **40**, 376-386.

164. Kato, J. and Katayama, T. (2001) Hda, a novel DnaA-related protein, regulates the replication cycle in *Escherichia coli*. *The EMBO Journal*, **20**, 4253-4262.

165. Camara, J.E., Skarstad, K. and Crooke, E. (2003) Controlled Initiation of Chromosomal Replication in Escherichia coli Requires Functional Hda Protein. *J Bacteriol*, **185**, 3244-3248.

166. Katayama, T. (2001) Feedback controls restrain the initiation of Escherichia coli chromosomal replication. *Mol Microbiol*, **41**, 9-17.

167. Nishida, S., Fujimitsu, K., Sekimizu, K., Ohmura, T., Ueda, T. and Katayama, T. (2002) A nucleotide switch in the *E. coli* DnaA protein initiates chromosomal replication: Evidence from a mutant DnaA protein defective in regulatory ATP hydrolysis in vitro and in vivo. *J Biol Chem*, **277**, 14986-14995.

168. Kurz, M., Dalrymple, B., Wijffels, G. and Kongsuwan, K. (2004) Interaction of the sliding clamp beta-subunit and Hda, a DnaA-related protein. *J Bacteriol*, **186**, 3508-3515.

169. Stock, A.M., Robinson, V.L. and Goudreau, P.N. (2000) Two-component signal transduction. *Annual Review of Biochemistry*, **69**, 183-215.

170. West, A.H. and Stock, A.M. (2001) Histidine kinases and response regulator proteins in two-component signaling systems. *Trends Biochem Sci*, **26**, 369-376.

171. Magasanik, B. (1988) Reversible phosphorylation of an enhancer binding protein regulates the transcription of bacterial nitrogen utilization genes. *Trends Biochem Sci*, **13**, 475-479.

172. Reitzer, L.J. and Magasanik, B. (1985) Expression of glnA in Escherichia coli is regulated at tandem promoters. *Proc Natl Acad Sci U S A*, **82**, 1979-1983.

173. Ueno-Nishio, S., Mango, S., Reitzer, L.J. and Magasanik, B. (1984) Identification and regulation of the glnL operator-promoter of the complex glnALG operon of Escherichia coli. *J Bacteriol*, **160**, 379-384.

174. Weiss, V., Claverie-Martin, F. and Magasanik, B. (1992) Phosphorylation of nitrogen regulator I of *Escherichia coli* induces strong cooperative binding to DNA essential for activation of transcription. *Proc. Natl. Acad. Sci. USA*, **89**, 5088-5092.

175. Mettke, I., Fiedler, U. and Weiss, V. (1995) Mechanism of activation of a response regulator: interaction of NtrC-P dimers induces ATPase activity. *J Bacteriol*, **177**, 5056-5061.

176. Morgan, D.O. (1997) *Annual Review of Cellular and Developmental Biology*, **13**, 261-291.

177. Nurse, P. and Bisssett, Y. (1981) *Nature*, **292**, 558-560.

178. Hartwell, L.H. (1971) *Journal of Molecular Biology*, **59**, 183-194.

179. Fisher, D.L. and Nurse, P. (1996) *EMBO Journal*, **15**, 850-860.

180. Brown, G.W., Jallepalli, P.V., Huneycutt, B.J. and Kelly, T.J. (1997) Interaction of the S phase regulator cdc18 with cyclin-dependent kinase in fission yeast. *Proc. Natl. Acad. Sci. USA*, **94**, 6142-6147.

181. Jallepalli, P.V., Brown, G.W., Muzi-Falconi, M., Tien, D. and Kelly, T.J. (1997) *Genes and Development*, **11**, 2767-2779.

182. Lopez-Girona, A., Mondesert, O., Leatherwood, J. and Russell, P. (1998) *Molecular Biology of the Cell*, **9**, 63-73.

183. Hendrickson, M., Madiine, M., Dalton, S. and Gautier, J. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 12223-12228.

184. Chong, J.P., Mahbubani, H.M., Khoo, C.Y. and Blow, J.J. (1995) Purification of an MCM-containing complex as a component of the DNA replication licensing system. *Nature*, **375**, 418-421.

185. Madine, M.A., Khoo, C.Y., Mills, A.D. and Laskey, R.A. (1995) MCM3 complex required for cell cycle regulation of DNA replication in vertebrate cells. *Nature*, **375**, 421-424.

186. Dahmann, C., Diffley, J.F. and Nasmyth, K.A. (1995) S-phase-promoting cyclin-dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state. *Curr Biol*, **5**, 1257-1269.

187. Diffley, J.F. (1996) Once and only once upon a time: specifying and regulating origins of DNA replication in eukaryotic cells. *Genes Dev*, **10**, 2819-2830.

188. Leatherwood, J., Lopez-Girona, A. and Russell, P. (1996) *Nature*, **379**, 360-363.

189. Ogawa, Y., Takahashi, T. and Masukata, H. (1999) *Mol. Cell. Biol.*, **19**, 7228-7236.

190. Sclafani, R.A. and Jackson, A.L. (1994) *Molecular Microbiology*, **11**, 805-810.

191. Yoon, H.-J. and Campbell, J.L. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3574-3578.

192. Jackson, A.L., Pahl, P.M., Harrison, K., Rosamond, J. and Sclafani, R.A. (1993) *Mol. Cell. Biol.*, **13**, 2899-2908.

193. Dowell, S.J., Romanowski, P. and Diffley, J.F.X. (1994) *Science*, **265**, 1243-1246.

194. Lei, M., Kawasaki, Y., Young, M.R., Kihara, M., Sugino, A. and Tye, B.K. (1997) *Genes and Development*, **11**, 3365-3374.

195. Hardy, C.F., Dryga, O., Seematter, S., Pahl, P.M. and Sclafani, R.A. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 3151-3155.

196. Alfano, C. and McMacken, R. (1989) Ordered assembly of nucleoprotein structures at the bacteriophage A replication origin during the initiation of DNA replication. *Journal of Biological Chemistry*, **264**, 10699-10708.

197. Zylicz, M., Gorska, L., Taylor, I.C. and Georgopoulos, C. (1988) Bacteriophage A replication proteins: formation of a mixed oligomer and binding to the origin of A DNA. *Mol. Gen. Genet.*, **196**, 401-406.

198. Taylor, K. and Wegrzyn, G. (1995) Replication of Coliphage Lambda DNA. *FEMS Microbiology Reviews*, **17**, 109-119.

199. Wegrzyn, G., Wegrzyn, A., Konieezny, I., Bielawski, K., Konopa, G., Obuehowski, M., Helinski, D.R. and Taylor, K. (1995) Involvement of the host initiator function *dnaA* in the replication of coliphage lambda. *Genetics*, **139**, 1469-1481.

200. Wegrzyn, G., Szalewska-Palasz, A., Wegrzyn, A., Obuehowski, M. and Taylor, F. (1995) Transcriptional activation of the origin of coliphage A DNA replication is regulated by the host DnaA initiator function. *Gene*, **154**, 47-50.

201. Georgupoulos, C. (1992) The emergence of the chaperone machines. *Trends in Biochemical Science*, **17**, 295-299.

202. Georgopoulos, C. and Welch, W. (1993) Role of major heat shock proteins as molecular chaperones. *Annual Review of Cell Biology*, **9**, 601-635.

203. Friedman, D.I., Olson, E.R., Tiily, K., Georgopoulos, C., Herskowitz, I. and Banuett, F. (1984) Interactions of baeteriophage lambda and host macromolecules in the growth of bacteriophage lambda. *Microbiology Reviews*, **48**, 299-325.

204. Zylicz, M., Aug, D., Liberek, K. and Georgopoulos, C. (1989) Initiation of lambda DNA replication with purified host-and bacteriophage-encoded proteins: the role of the DnaK, DnaJ and GrpE heat shock proteins. *EMBO J.*, **8**, 1601-1608.

205. Hendrick, L.a.H., F.-U. (1993) Molecular chaperone functions of heat-shock proteins. *Annual Review of Biochemistry*, **62**, 349-384.

206. Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C. and Zylicz, M. (1991) The *Escfierichia coli'DnaJ* and GrpE heat shock proteins jointly stimulate DnaK's ATPase activity. *Proc. Natl. Acad. Sci. USA*, **88**, 2874-2878.

207. Langer, T., Lu, C., Eehols, H., Flanagan, J., Hayer, M.K. and Hartl, F.-U. (1992) Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. *Nature*, **356**, 683-689.

208. Palleros, D.R., Reid, K.L., Shi, L., Welch, W.J. and Fink, A.L. (1993) ATP-induced protein-HspT0 complex dissociation requires K + but not ATP hydrolysis. *Nature*, **365**, 664-666.

209. Osipiuk, J., Georgopoulos, C. and Zylicz, M. (1993) Initiation of A DNA replicalion: the *Escherichia coil* small heatshock proteins, DnaJ and GrpE, increase DnaK's affinity for the lambda-P protein. *Journal of Biological Chemistry*, **268**, 4821-4827.

210. Kobiler, O., Oppenheim, A.B. and Herman, C. (2004) Recruitment of host ATP-dependent proteases by bacteriophage lambda. *Journal of Structural Biology*, **146**, 72-78.

211. Gottesman, S., Clark, W.P., de Crecy-Lagard, V. and Maurizi, M.R. (1993) ClpX, an alternative subunit for the ATP-dependent ClpP protease of *Escherichia coli.* Sequence and *in vivo* activities. *Journal of Biological Chemistry*, **268**, 22618-22626.

212. Czyz, A., Zielke, R. and Wegrzyn, G. (2001) Rapid degradation of bacteriphage lambda O protein by ClpP/ClpX protease influences the lysis-versus-

lysogenization decision of the phage under certain growth conditions of the host cells. *Archives of Virology*, **146**, 1487-1498.

213. Ortega, J., Singh, S.K., Ishikawa, T., Maurizi, M.R. and Steven, A.C. (2000) Visualization of substrate binding and translocation by the ATP-dependent protease, ClpXP. *Molecular Cell*, **6**, 1515-1521.

214. Ortega, J., Lee, H.S., Maurizi, M.R. and Steven, A.C. (2002) Alternating translocation of protein substrates from both ends of ClpXP protease. *EMBO J*, **21**, 4938-4949.

215. Chaconas, G., Harshey, R.M., Sarvetnick, N. and Bukhari, A.I. (1981) Predominant end-products of prophage Mu DNA transposition during the lytic cycle are replicon fusions. *Journal of Molecular Biology*, **150**, 341–359.

216. Burton, B.M. and Baker, T.A. (2006) Remodeling protein complexes: Insights from the AAA+ unfoldase ClpX and Mu transposase. *Protein Science*, **14**, 1945-1954.

217. Nakai, H. and Kruklitis, R. (1995) Disassembly of the bacteriophage Mu transposase for the initiation of Mu DNA replication. *Journal of Biological Chemistry*, **270**, 19591–19598.

218. Levchenko, I., Luo, L. and Baker, T.A. (1995) Disassembly of the Mu Transposase Tetramer by the ClpX Chaperone. *Genes and Development*, **9**, 2399-2408.

219. Mhammedi-Alaoui, A., Pato, M., Gama, M.J. and Toussaint, A. (1994) A new component of bacteriophage Mu replicative transposition machinery: The Escherichia coli ClpX protein. *Molecular Microbiology*, **11**, 1109–1116.

220. Kruklitis, R., Welty, D.J. and Nakai, H. (1996) ClpX protein of Escherichia coli activates bacteriophage Mu transposase in the strand transfer complex for initiation of Mu DNA synthesis. *EMBO Journal*, **15**, 935-944.

221. Burton, B.M. and Baker, T.A. (2003) Mu Transpososome Architecture Ensures that Unfolding by ClpX or Proteolysis by ClpXP Remodels but Does Not Destroy the Complex. *Chem Biol*, **10**, 463-472.

222. Wickner, S., Hoskins, J. and McKenney, K. (1991) Function of DnaJ and DnaK as chaperones in origin-specific DNA binding by RepA. *Nature*, **350**, 165-167.

223. Wickner, S., Hoskins, J. and McKenney, K. (1991) Monomerization of RepA Dimers by Heat Shock Proteins Activates Binding to DNA Replication Origin. *Proc. Natl. Acad. Sci. USA*, **88**, 7903-7907.

224. Chung, C.H. (1993) Proteases in Escherichia coli. *Science*, **262**, 372-374.

225. Gottesman, S. (1996) Proteases and their targets in *Escherichia coli*. *Annual Review of Genetics*, **30**, 465-506.

226. Wickner, S., Gottesman, S., Skowyra, D., Hoskins, J., McKenney, K. and Maurizi, M.R. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 12218–12222.

227. Schmidhauser, T.J. and Helinski, D.R. (1985) Regions of the Broad-Host -Range Plasmid RK2 Involved in Replication and Maintenance in Nine Species of Gram-Negative Bacteria. *Journal of Bacteriology*, **164**, 446-455.

228. Figurski, D.H. and Helinski, D.R. (1979) Replication of an Oigin-Containing Derivative of Plasmid RK2 Dependent on a Plasmid Function Provided in *trans*. *Proc. Natl. Acad. Sci. USA*, **76**, 1648-1652.

229. Thomas, C.M., Meyer, R. and Helinski, D.R. (1980) Regions of Broad-Host-Range Plasmid RK2 Which are Essential for Replication and Maintenance. *Journal of Bacteriology*, **141**, 213-222.

230. Gaylo, P.J., Turjman, N. and Bastia, D. (1987) DnaA protein is required for replication of the minimal replicon of the broad-host-range plasmid RK2 in Escherichia coli. *J Bacteriol*, **169**, 4703-4709.

231. Pinkney, M., Diaz, R., Lanka, E. and Thomas, C.M. (1988) Replication of mini RK2 plasmid in extracts of Escherichia coli requires plasmid-encoded protein TrfA and host-encoded proteins DnaA, B, G DNA gyrase and DNA polymerase III. *J Mol Biol*, **203**, 927-938.

232. Konieczny, I., Doran, K.S., Helinski, D.R. and Blasina, A. (1997) Role of TrfA and DnaA Proteins in Origin Opening during Initiation of DNA Replication of the Broad Host Range Plasmid RK2. *The Journal of Biological Chemistry*, **272**, 20173-20178.

233. Perri, S., Helinski, D.R. and Toukdarian, A. (1991) Interactions of plasmid-encoded replication initiation proteins with the origin of DNA replication in the broad host range plasmid RK2. *J Biol Chem*, **266**, 12536-12543.

234. Konieczny, I. and Helinski, D.R. (1997) The replication initiation protein of the broad-host-range plasmid

RK2 is activated by the ClpX chaperone. *PNAS*, **94**, 14378-14382.

235. Marczynski, G.T. and Shapiro, L. (2002) Control of chromosome replication in *Caulobacter crescentus*. *Annual Reviews in Microbiology*, **56**, 625-656.

236. Ausmees, N., Kuhn, J.R. and Jacobs-Wagner, C. (2003) The bacterial cytoskeleton. An intermediate filament-like function in cell shape. *Cell*, **115**, 705-713.

237. Shapiro, L. and Losick, R. (2000) Dynamic spacial regulation in the bacterial cell. *Cell*, **100**, 89-98.

238. Poindexter, J.S. (1981) The *Caulobacters*: Ubiquitous unusual bacteria. *Microbiological Reviews*, **45**, 123-179.

239. Nierman, W.C., Feldblyum, T.V., Laub, M.T., Paulsen, I.T., Nelson, K.E., Eisen, J., Heidelberg, J.F., Alley, M.R.K., Ohta, N., Maddock, J.R. *et al.* (2001) Complete genome sequence of *Caulobacter crescentus*. *Proc. Natl Acad. Sci. USA*, **98**, 4136-4141.

240. Laub, M.T., McAdams, H.H., Fraser, C.M. and Shapiro, L. (2000) Global analysis of the genetic network controlling a bacterial cell cycle. *Science*, **290**, 2144-2148.

241. Grunenfelder, B., Rummel, G., Vohradsky, J., Roder, D., Langen, H. and Jenal, U. (2001) Proteomic analysis of the bacterial cell cycle. *Proc. Natl. Acad. Sci. USA*, **98**, 4681-4686.

242. Wright, R., Stephens, C., Zweiger, G., Shapiro, L. and Alley, M.R.K. (1996) *Caulobacter* Lon protease has a critical role in cell-cycle control of DNA methylation. *Genes & Development*, **10**, 1532-1542.

243. Fischer, B., Rummel, G., Aldridge, P. and Jenal, U. (2002) The FtsH protease is involved in development, stress response and heat shock control in Caulobacter crescentus. *Mol Microbiol*, **44**, 461-478.

244. Jenal, U. and Fuchs, T. (1998) An essential protease involved in bacterial cell cycle control. *The EMBO Journal*, **17**, 5658-5669.

245. Crosson, S., McAdams, H. and Shapiro, L. (2004) A genetic oscillator and the regulation of cell cycle progression in *Caulobacter crescentus*. *Cell Cycle*, **3**, 55-58.

246. Quon, K.C., Yang, B., Domian, I.J., Shapiro, L. and Marczynski, G.T. (1998) Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin. *Proc. Natl. Acad. Sci. USA*, **95**, 120-125.

247. Quon, K.C., Marczynski, G.T. and Shapiro, L. (1996) Cell cycle control by an essential bacterial two-component signal transduction protein. *Cell*, **84**, 83-93.

248. Holtzendorff, J., Hung, D., Brende, P., Reisenauer, A., Viollier, P.H., McAdams, H.H. and Shapiro, L. (2004) Oscillating Global Regulators Control the Genetic Circuit Driving a Bacterial Cell Cycle. *Science*, **304**, 983-987.

249. Ohta, N., Grebe, T.W. and Newton, A. (2000) In Shimkets, L. J. and Brun, Y. V. (eds.), *Prokaryotic Development*. American Society for Microbiology, Washington, DC, pp. 341-359.

250. Wheeler, R.T. and Shapiro, L. (1999) Differential localization of two histidine kinases controlling bacterial cell differentiation. *Molecular Cell*, **4**, 683-694.

251. Guillet, V., Ohta, N., Cabantous, S., Newton, A. and Samama, J.P. (2002) Crystallographic and biochemical studies of DivK reveal novel features of an essential response regulator in Caulobacter crescentus. *J Biol Chem*, **277**, 42003-42010.

252. Jacobs, C., Hung, D. and Shapiro, L. (2001) Dynamic localization of a cytoplasmic signal transduction response regulator controls morphogenesis during the Caulobacter cell cycle. *Proc. Natl. Acad. Sci. USA*, **98**, 4095-4100.

253. Wang, S.P., Sharma, P.L., Shoenlein, P.V. and Ely, B. (1993) A histidine protein kinase is involved in polar organelle development in *Caulobacter crescentus*. *Proc. Natl. Acad. Sci. USA*, **90**, 630-634.

254. Skerker, J.M. and Shapiro, L. (2000) Identification and cell cycle control of a novel pilus system in *Caulobacter crescentus*. *The EMBO Journal*, **19**, 3223-3234.

255. Viollier, P.H., Sternheim, N. and Shapiro, L. (2002) A dynamically localized histidine kinase controls the asymmetric distribution of polar pili proteins. *EMBO Journal*, **21**, 4420-4428.

256. Viollier, P.H., Sternheim, N. and Shapiro, L. (2002) Identification of a localization factor for the polar positioning of bacterial structural and regulatory proteins. *Proc Natl Acad Sci U S A*, **99**, 13831-13836.

257. Hinz, A.J., Larson, D.E., Smith, C.S. and Brun, Y.V. (2003) The *Caulobacter crescentus* polar organelle development protein PodJ is differentially localized and is required for polar targeting of the PleC development regulator. *Mol Microbiol*, **47**, 929-941.

258. Muir, R.E. and Gober, J. (2001) Regulation of late flagellar gene transcription and cell division by flagellum assembly in *Caulobacter crescentus*. *Molecular Microbiology*, **41**, 117-130.

259. Muir, R.E. and Gober, J.W. (2002) Mutations in FlbD that relieve the dependency on flagellum assembly alter the temporal and spatial pattern of developmental transcription in Caulobacter crescentus. *Molecular Microbiology*, **43**, 597-615.

260. Mohr, C., MacKichan, J.K. and Shapiro, L. (1998) A Membrane-Associated Protein, FliX, Is required for an Early Step in *Caulobacter* Flagellar Assembly. *Journal of Bacteriology*, **180**, 2175-2185.

261. Muir, R.E., M, O.B.T. and Gober, J. (2001) The *Caulobacter crescentus* flagellar genes, *fliX*, encodes a novel *trans*-acting factor that couples flagellar assembly to transcription. *Molecular Microbiology*, **39**, 1623-1637.

262. Muir, R.E. and Gober, J.W. (2004) Regulation of FlbD activity by flagellum assembly is accomplished through direct interaction with the trans-acting factor, FliX. *Mol Microbiol*, **54**, 715-730.

263. Muir, R.E. and Gober, J.W. (2005) Role of integration host factor in the transcriptional activation of flagellar gene expression in Caulobacter crescentus. *J Bacteriol*, **187**, 949-960.

264. Mohl, D.A. and Gober, J.W. (1997) Cell Cycle-Dependent Polar Localization of Chromosome Partitioning Proteins in *Caulobacter crescentus*. *Cell*, **88**, 675-684.

265. Easter, J. and Gober, J. (2002) ParB-Stimulated Nucleotide Exchange Regulates a Switch in Functionally Distinct ParA Activities. *Mol Cell*, **10**, 427.

266. Jensen, R.B. and Shapiro, L. (1999) The *Caulobacter crescentus smc* gene is required for cell cycle progression and chromosome segregation. *Proc. Natl. Acad. Sci. USA*, **96**, 10661-10666.

267. van den Ent, F., Amos, L.A. and Lowe, J. (2001) Prokaryotic origin of the actin cytoskeleton. *Nature*, **413**, 39-44.

268. Jones, L.J.F., Carballido-Lopez, R. and Errington, J. (2001) Control of cell shape in bacteria: Helical, actin-like filaments in *Bacillus subtilis*. *Cell*, **104**, 913-922.

269. Kruse, T., Moller-Jensen, J., Lobner-Olesen, A. and Gerdes, K. (2003) Dysfunctional MreB inhibits chromosome segregation in Escherichia coli. *EMBO J*, **22**, 5283-5292.

270. Figge, R.M., Divakaruni, A.V. and Gober, J.W. (2004) MreB, the cell shape-determining bacterial actin homologue, co-ordinates cell wall morphogenesis in *Caulobacter crescentus*. *Mol Microbiol*, **51**, 1321-1332.

271. Gitai, Z., Dye, N. and Shapiro, L. (2004) An actin-like gene can determine cell polarity in bacteria. *Proc Natl Acad Sci U S A*, **101**, 8643-8648.

272. Gitai, Z., Dye, N.A., Reisenauer, A., Wachi, M. and Shapiro, L. (2005) MreB Actin-Mediated Segregation of a Specific Region of a Bacterial Chromosome. *Cell*, **120**, 329-341.

273. Kruse, T. and Gerdes, K. (2005) Bacterial DNA segregation by the actin-like MreB protein. *Trends in Cell Biology*, **15**, 343-345.

274. Marczynski, G.T. and Shapiro, L. (1992) Cell-cycle Control of a Cloned Chromosomal Origin of replication from *Caulobacter crescentus*. *Journal of Molecular Biology*, **226**, 959-977.

275. Brassinga, A.K.C. and Marczynski, G.T. (2001) Replication intermediate analysis confirms that chromosome replication initiates from an unusual intergenic region in *Caulobacter crescentus*. *Nucleic Acids Research*, **29**, 4441-4451.

276. Marczynski, G.T., Lentine, K. and Shapiro, L. (1995) A developmentally regulated chromosomal origin of replication uses essential transcription elements. *Genes and Development*, **9**, 1543-1557.

277. Brassinga, A.K.C., Siam, R. and Marczynski, G.T. (2001) Conserved gene cluster at replication origins of the alpha-proteobacteria *Caulobacter crescentus* and *Rickettsia prowazekii*. *Journal of Bacteriology*, **183**, 1824-1829.

278. Bates, D.B., Boye, E., Asai, T. and Kogoma, T. (1997) The Absence of an Effect of *gid* or *mioC* Transcription on the Initiation of Chromosome Replication in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*, **94**, 12497-12502.

279. DePamphilis, M.L. (1993) Eukaryotic DNA Replication: Anatomy of An Origin. *Annual Review in Biochemistry*, **62**, 29-63.

280. Messer, W. and Weigel, C. (1996) In Neidhardt, F. C., Curtiss III, R., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M. and Umbarger, H. E. (eds.), *Escherichia coli and Salmonella, Cellular and Molecular Biology*. ASM Press, Washington, D.C., Vol. 1, pp. 1579-1601.

281. Domian, I.J., Reisenauer, A. and Shapiro, L. (1999) Feedback control of a master bacterial cell cycle regulator. *Proc. Natl. Acad. Sci. USA*, **96**, 6648-6653.

282. Domian, I.J., Quon, K.C. and Shapiro, L. (1997) Cell type-specific phosphorylation and proteolysis of a transcriptional regulator controls the G1 to S transition in a bacterial cell cycle. *Cell*, **90**, 415-424.

283. Fuchs, T.M., Deppisch, H., Scarlato, V. and Gross, R. (1996) A new gene locus of Bordetella pertussis defines a novel family of prokaryotic transcriptional accessory proteins. *J Bacteriol*, **178**, 4445-4452.

284. Reisenauer, A., Quon, K. and Shapiro, L. (1999) The CtrA response regulator mediates temporal control of gene expression during the *Caulobacter* cell cycle. *Journal of Bacteriology*, **181**, 2430-2439.

285. Gorbatyuk, B. and Marczynski, G.T. (2001) Physiological consequences of blocked *Caulobacter crescentus* DnaA expression, an essential DNA replication gene. *Molecular Microbiology*, **40**, 485-497.

286. Gorbatyuk, B. and Marczynski, G.T. (2005) Regulated degradation of chromosome replication proteins DnaA and CtrA in Caulobacter crescentus. *Mol Microbiol*, **55**, 1233-1245.

287. Stephens, C.M., Reisenauer, A., Wright, R. and Shapiro, L. (1996) A cell cycle-regulated bacterial DNA methyltransferase is essential for viability. *Proc. Natl. Acad. Sci. USA*, **93**, 1210-1214.

288. Stephens, C.M., Zweiger, G. and Shapiro, L. (1995) Coordinate cell cycle control of a *Caulobacter* DNA methyltransferase and the flagellar genetic hierarchy. *Journal of Bacteriology*, **177**, 1662-1669.

289. Reisenauer, A., Jensen, R.B. and Shapiro, L. (2002) DNA methylation affects the cell cycle transcription of the CtrA global regulator in *Caulobacter*. *The EMBO Journal*, **21**, 4969-4977.

290. Marczynski, G.T. (1999) Chromosome methylation and the measurement of faithful, once and only once per cell cycle chromosome replication in *Caulobacter crescentus*. *Journal of Bacteriology*, **181**, 1984-1993.

291. Zweiger, G., Marczynski, G.T. and Shapiro, L. (1994) A *Caulobacter* DNA methyltransferase that functions only in the predivisional cell. *Journal of Molecular Biology*, **235**, 472-485.

292. Blow, J.J. and Tada, S. (2000) A new check on issuing licence. *Nature*, 560-561.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

Chapter 2

1. The response regulator CtrA binding affinity to two *ctrA* promoters is independent of CtrA phosphorylation. This result identifies a novel mode of binding for this large class of proteins.
2. Developed a sensitive chromatin immunoprecipitation assay to demonstrate that CtrA binds the *ctrA* and *motB* promoters *in vivo*.
3. Expression of a non-proteolyzable CtrA allele (CtrAΔ3) reveals constitutive binding to these promoters.
4. Continuous occupation of the *ctrA* promoter by CtrA does not alter the cell cycle activity of this promoter and suggests a new allosteric model for CtrA regulated transcription.

Chapter 3

1. Direct demonstration that CtrA binds to the *Caulobacter* replication origin *in vivo*. CtrA does not occupy *Cori* in stalk cells when replication commences.
2. In contrast to *ctrA*-regulated promoters, the CtrAΔ3 allele does not constitutively occupy *Cori* in cell cycle experiments.
3. The chaperone ClpX is recruited to *Cori* prior to start of S-phase.
4. Increased CtrA binding to *Cori* stimulates increased ClpX recruitment.

Chapter 4

1. Identified and cloned two DnaA-like genes (Cdl-1 and Cdl-2).
2. Cdl-1 is a homolog of the DnaA ATPase domain and similar to a component of Regulated Inhibition of DnaA (RIDA) found in *E. coli.*
3. Cdl-2 is homologous to the DNA binding domain of DnaA and is restricted to the apha-proteobacter class of Gram negative bacteria.
4. Over expression of Cdl-1 or Cdl-2 in *Caulobacter* causes morphological defects and a failure to divide. Overexpression also inhibits DNA synthesis

TRANSITION I

The section on *Caulobacter* chromosome replication, outlined in the literature review, introduces CtrA as a major regulator of chromosome replication. Work performed by. Rania Siam, a previous Ph.D. candidate in our lab, demonstrated that right side or left side disruptions in CtrA binding sites retained a very weak affinity for CtrA protein. However, increased affinity for these mutated binding sites could not be stimulated by phosphorylation. Analysis of the *Caulobacter* genome reveals that the *ctrA* promoters (and others) have binding sites that do not reflect the proposed consensus sequence with 7 bp spacing, namely TTAA-N7-TTAA. This variation suggests CtrA's role as a transcription factor may differ from its role as a replication inhibitor because the type of bindings sites utilized are not similar. The immediate question was to test how binding to the *ctrA* promoter was influenced by phosphorylation. The absence of increased affinity of CtrA-phosphate for these binding sites was unexpected, because well studied response regulators show an increased affinity for target DNA upon phosphorylation. As will be discussed, the novel behaviour of CtrA at its own promoter challenges the recruitment model of transcription and introduces a simple allosteric model to encompass new biochemical activities. It is from these beginnings that the remaining chapters were developed in the hope of understanding how CtrA and newly emerging mechanisms negatively control DNA replication in *Caulobacter*.

Chapter II: Phosphorylation does not stimulate DNA binding of the response regulator CtrA to a new class of *Caulobacter crescentus* promoters

Will Spencer, Rania Siam, Marie-Claude Ouimet, and Gregory T. Marczynski*

Address: Department of Microbiology and Immunology
McGill University
3775 University Street
Montreal, Quebec, Canada
H3A 2B4
(Tel) 514-398-3917
(Fax) 514-398-7052
email: gregory.marczynski@mcgill.ca

*- corresponding author

ABSTRACT

Response regulator proteins typically gain affinity for their target DNA upon phosphorylation. Likewise, the *Caulobacter crescentus* cell-cycle transcription regulator CtrA gains affinity for consensus TTAA-N7-TTAA sites present at the replication origin and the promoters of many cell cycle expressed genes. Although transcription of the *ctrA* gene is auto-regulated by CtrA binding to two (P1 and P2) promoters, their TTAA motifs lack the consensus N7 spacing. We show that the P1 and P2 promoters have distinct CtrA binding sites with equal and low affinity ($K_d \sim 0.3$ μM) for CtrA and phosphorylated CtrA (CtrA~P). Unexpectedly, both *ctrA* promoters retain their cell-cycle programs of activation and repression when CtrA protein is present throughout the cell cycle. Under these conditions CtrA protein continuously occupies P1 and P2. We therefore propose that cell-cycle DNA binding is not the primary requisite for regulation and cell-cycle mediated CtrA phosphorylation drives transcription. Therefore, phosphorylation is not a mechanism for CtrA recruitment to P1 and P2 and despite the low affinity of these promoters, the effective *in vivo* concentration of CtrA is high (~10,000 copies/cell) so that these promoters can be occupied *in vivo*. This class of promoters may be common, since a whole genome survey of TTAA half sites showed a bias for the 5' ends of genes.

INTRODUCTION

Asymmetric cell division in the Gram-negative bacterium *Caulobacter crescentus* forms two distinct "swarmer" and "stalked" cell progeny (Figure 1) each possessing different replication and transcriptional programs (1). The motile swarmer cells must commit to stalked development prior to initiation of chromosome replication and a key component of this cell cycle checkpoint is the response regulator CtrA (2). CtrA is restricted to swarmer cells, following cell division, and is removed during the swarmer to stalk transition by targeted proteolysis (3). Previous models have suggested that inducible turnover of CtrA is a necessary step to alleviate repression of chromosome replication and promote stalk specific transcription. During chromosome replication CtrA is resynthesized and sequestered to the swarmer compartment of the predivisional cell while specific proteolysis limits CtrA in the stalk compartment (4). However, cell cycle regulated proteolysis of CtrA appears dispensable because a non-proteolyzable mutant of CtrA supports an apparently normal cell cycle (5). In addition to its role in replication, CtrA also functions to control the expression of numerous genes in *C. crescentus* (6). CtrA homologues have been identified in related bacteria and these may have a similar role as global cell cycle regulators (7-10).

CtrA protein synthesis requires transcription from two adjacent promoters, designated P1 and P2, each with a distinct pattern of cell cycle regulation (11). Following degradation and loss of CtrA~P in the stalked cell, selective derepression of the P1 promoter

replenishes CtrA. As CtrA levels increase in pre-divisional cells, a transition from P1 to P2-directed transcription occurs because CtrA activates P2 but represses P1.

CtrA belongs to a broad family of response regulators bearing homology to *Escherichia coli* OmpR (12) and like many response regulator proteins, it is the phosphorylation of CtrA that presumably coordinates cell cycle transcription and CtrA binding to the replication origin (*Cori*). Phosphorylated CtrA (CtrA~P) coincides with CtrA proteolysis (4). The essential histidine kinase, CckA, is an important aspect of CtrA activity (12) and functions either as a direct phospho-donor or within a much wider phospho-relay network. Other kinases that are not essential (DivJ and PleC) may also phosphorylate CtrA (13-15).

Within *Cori*, CtrA~P stimulates binding to five sites (a-e) having the consensus TTAA-N7-TTAA (N = any nucleotide) (16). It is believed that CtrA~P binding to *Cori* represents an important switch for regulating chromosome replication in swarmer cells (17). On the other hand, it is not clear whether this view of CtrA binding is accurate for the majority of CtrA-regulated promoters. A recent analysis of the intergenic regions of the *Caulobacter* genome reveals numerous target genes whose transcription is directly regulated by CtrA (6,18). However, sequence comparisons of the canonical CtrA consensus sequences found in *Cori* with these promoters reveals a surprising lack of conservation. Many predicted promoters have atypical CtrA binding sites, including variable spacing between half sites (N≠7) or promoters with only one half site (19). These results suggest that the TTAA N7 TTAA consensus may not account for all the CtrA binding sites at these promoters. The *ctrA* promoter demonstrates these atypical

features, comprising a single TTAA in the P1 promoter and three half sites in the P2 promoter (Figure 4A). The apparent difference between CtrA binding sites within *Cori* and within P1 and P2 prompted us to examine CtrA binding to these non-consensus sites.

In this study, we demonstrate that CtrA phosphorylation does not increase its affinity for the P1 and P2 promoters of the *ctrA* gene *in vitro*. Quantitation of CtrA protein copy number in cells demonstrates that CtrA is unexpectedly abundant. Over expression of a non-proteolyzable allele of CtrA (CtrAΔ3) demonstrates constitutive binding to the *ctrA* promoters *in vivo* but does not perturb the cell-cycle regulation of these promoters and suggests that binding is not the rate limiting step in controlling these promoters. We identify an ctrA-promoter like region upstream of *motB* and confirm by chromatin immunoprecipitation assays that CtrA binds this region in a cell cycle dependent manner similar to the *ctrA* promoter. These data suggest phosphorylation provides other biochemical properties aside from DNA binding that are important for regulating these promoters.

MATERIALS AND METHODS

Strains and Plasmids

All experiments were carried out using NA1000 (formerly CB15N), a synchronizable *Caulobacter* strain. All cultures were grown exponentially from an OD_{660} of 0.1 and maintained at 30°C in liquid M2 supplemented with 0.2% glucose. The promoter regions of the *ctrA* gene corresponding to P1 and P2 were cloned upstream of the *lacZ* gene in the pRK290*lacZ* plasmid (11) and mobilized into *Caulobacter* by conjugation with *Escherichia coli* strain S-17-1 (20). For DNase I foot printing assays, pTRC7.4 carrying a histidine-tagged CtrA fusion protein was overexpressed in *E. coli* BL21 cells and purified as previously described (2). The *ctrAΔ3* allele (4) was heterologously expressed from the xylose inducible promoter (PxylX) within the pUJ142 vector (21). However, for these experiments, CtrAΔ3 expressing cells were grown in glucose to promote only basal expression and thus avoid non-physiological over-expression of CtrA protein.

DNase I Footprint Assay

Dnase I footprints were carried out as previously described (16). Salmon sperm DNA (Sigma) and poly(dI-dC) (GE Healthcare) were used as non-specific DNA buffers and demonstrated no significant differences. DNA fragment corresponding to the P1 and P2 promoters of the *ctrA* gene was end labeled as previously described (2). CtrA phospholabelling reactions were carried out using purified EnvZ with or without ATP as previously described (16). Because CtrA~P has a relative half life of approximately 1h under assay conditions employed (data not shown) and ensured that binding assays could be effectively completed within the prescribed time (~10 min).

Immunoprecipitations

Synchronized cells of NA1000 with plasmids (+/- CtrAΔ3) containing either the P1::lacZ or P2::lacZ reporter plasmids (11) were pulse labeled with 15 uCi of 35S-Methionine for 10 minutes in PYE media at room temperature. Lysates were prepared by treatment with 10 mg/ml lysozyme in 50mM Tris pH 8, 450 mM NaCl, and 0.5% Triton X-100 (TNT) followed by preclearing with protein-A coated agarose beads (Sigma-Aldrich). Approximately $2x10^6$ cpm of lysate and 0.5ul of polyclonal rabbit anti–β galactosidase antibody (Sigma-Aldrich) was used for immunoprecipitation. Cells were washed three times with TNT buffer and the resulting pellet resuspended in 4 volumes of SDS protein loading buffer (2% SDS, 2% β-mercaptoethanol). Immunoprecipitated lysates were resolved on 10% SDS PAGE gels and analyzed by phosphorimager (Molecular Dynamics).

Electromobility Shift Assays

An EcoRI/HindIII fragment of a test promoter (22) carrying an intact CtrA binding site (pGM1871 – TTAA-N7-TTAA) or a mutated half site (pGM1713 – TTAA-N7-TCAG) were dephosphorylated with calf-intestinal phosphatase (GE Healthcare) and purified by gel isolation (Qiagen). Fragments were labeled with 0.25 μCi of gamma-32P-ATP for 10 minutes (37°C) in the presence of poly-nucleotide kinase (GE Healthcare) and commercially supplied PNK buffer. Reactions were stopped with 2 μl 0.5M EDTA and incubated with Na-acetate, oyster glycogen and TE buffer. Samples were precipitated

with ice cold absolute ethanol and stored at -20°C for 30 minutes. Pellets were collected by centrifugation and washed with 70% ethanol and resuspended in 30 µl of TE buffer. 1 µl aliquots were counted by liquid scintillation (Canberra Tri Carb) and approximately 20,000 cpm of labeled DNA were used per binding reaction. For EMSAs, histidine tagged- (1mg/ml) or GST tagged- (4mg/ml) CtrA protein were used separately or in combination for probe binding. Protein-DNA complexes were resolved using 5% tris-glycine native polyacrylamide gel electrophoresis, vacuum dried on Whatmann 3MM and incubated with autoradiography film (Kodak Biomax MR-1) at -80°C overnight.

CtrA Western blotting

CtrA western blots were performed using standard SDS-PAGE conditions with transfer to PVDF (HyBond, Amersham Biosciences). Membranes were blocked in 5% non-fat skim milk in Tris buffered saline (TBS) and immunoblots were carried out using a 1:5000 dilution of the CtrA rabbit polyclonal primary antibody, provided by Lucy Shapiro, Stanford California (17)and a 1:10,000 dilution of the goat anti rabbit secondary (Chemicon). Membranes were washed repeatedly in fresh TBS buffer and developed using the West Pico Chemiluminescent substrate kit (Pierce).

In vivo Quantitation of CtrA

We determined the number of CtrA protein molecules per cell in both synchronized and unsynchronized cultures using western blots of cell lysates titrated across the linear range for chemiluminscent detection (ECL: Amersham Biosciences) and referenced against known concentrations of purified CtrA. We quantified our purified preparation of CtrA

by spectrophotometric analysis (BCA: Pierce) using the manufacturers supplied protein standard (albumin – 2 mg/ml) as a reference. Westerns blots were developed using Kodak MR-1 film and images were analyzed using the Scion Image software package and the GelPlot2 macros (www.scioncorp.com) to quantitate CtrA in prepared lysates.

Chromatin Immunoprecipitation Assay (ChIP)

This assay procedure was performed as previously described (6) with the noted exceptions. Cell lysis was carried out using BugBuster reagent as specified by manufacturer (Novagen). A 157 bp fragment overlapping the P1 and P2 promoters of the *ctrA* promoter was PCR amplified from serial dilutions (1:1, 1:2, 1:4, 1:8, 1:16, 1:32) of immunoprecipitated and mock-immunoprecipitated DNA using the following primers

Table 1 – PCR primers used for ChIP Assay

CTRApromoter1	(5'-CGCTGTCATCCTCGATCAAC-3')
CTRApromoter2	(5'-CTCCGACGGGAAACATTCAC-3')
MotB-forward	(5'-AGGATGCCGCCGG-3')
MotB-reverse	(5'-CCGTCTTCATGCCGC-3')

PCR was carried out using the FailSafe PCR premix system (Epicenter) and purified Taq polymerase (reference). PCR products were run on 1% agarose, scanned and quantitated using the GelDoc System (check name) (Kodak).

CtrA half-site consensus analysis

To determine the distribution of CtrA half site consensus motifs (TTAA) a searchable text string was generated within Microsoft Word™ to manipulate the entire genome sequence of Caulobacter obtained from TIGR (http://www.tigr.org/tigr-

scripts/CMR2/CMRHomePage.spl). Using the integrated search function we were able to identify and label all TTAA motifs within the genome. We then identified pairs of half sites whose intervening sequence were less than 20 base pairs. We set the upper limit of our half site spacing to 20 bp because values greater than 20 bp would unlikely play any cooperative role in CtrA binding. We gathered positional information about these half sites, by mapping their relative positions to adjacent open reading frames using BLASTN and the intergenic regions confirmed using GENOME-TOOLS (http://caulo.stanford.edu/genome-tools/).

RESULTS

CtrA binding to consensus half-sites is unstable.

For the best studied response regulators systems, the functional unit comprises a protein dimer complexed to a consensus DNA sequence formed from two separate half-sites, one for each protein monomer. For example, OmpR, a response regulator that controls osmo-regulation in *E. coli*, binds to four 20 bp consensus sequences (F1-F4) to repress expression of the outermembrane porin *ompF* (23). OmpR interacts with its consensus sequence as a dimer. Prior to phosphorylation, monomer-half site interactions are weak and phosphorylation stabilizes the protein dimer interface to increases DNA binding (24,25).

Phosphorylation of CtrA demonstrates two distinct modes of DNA binding at the *Caulobacter* replication origin; 1) increased affinity for one target site and 2) cooperativity between two adjacent target sites (16). *In vitro* DNase I footprint assays of *Cori* revealed that CtrA could still recognize, although weakly, binding sites carrying a disruption in one of the half sites. However, binding to the remaining half-site was not stimulated by phosphorylation. This represents a third mode of CtrA binding *in vitro* and we began to look for CtrA regulated promoters that might utilize this mode of binding *in vivo*.

We tested whether CtrA binding was dependent on dimer formation or, in the case of half sites, binding could be facilitated by monomers. We tested this behaviour using an established CtrA-regulated test promoter that functions *in vivo* (22). We initially tested

dimer formation using mixtures of HIS-CtrA and GST-CtrA protein fusions (Figure 2). *In vitro* we anticipated that dimer formation in a mixture of HIS-CtrA and GST-CtrA would generate three possible dimer species that should be distinguishable by DNA mobility-shift assays because each complex gives a distinct gel shift (see Figure 2A). In Figure 2B, lane one shows the unbound test promoter and lanes 2-10 used a fixed concentration of His6-CtrA (1 mg/ml) titrated with an increasing concentration of purified GST-CtrA (0.01 mg/ml – 2 mg/ml). Lanes 11 and 12 corresponded to the shifted probe using only His6-CtrA or GST-CtrA as controls. Our results indicate that GST-CtrA had unexpected high affinity for the test promoter and out competed HIS-CtrA at very low concentrations suggesting that the dimer equilibrium may not favor HIS-CtrA/GST-CtrA heterodimer formation. We next compared the affinity of GST-CtrA (high affinity) and HIS-CtrA (low affinity) to a test promoter carrying either an intact CtrA binding site or a mutant binding site (disrupted half site). The data show that neither HIS-CtrA (Figure 2C lanes 1-3) nor GST-CtrA (Figure 2C, lanes 4-6) interact with the mutated half site over all concentrations tested even though the test promoter, carrying the intact CtrA binding site, was shifted by both forms of CtrA at much lower protein concentrations (Figure 2C, lanes 7 & 8).

CtrA consensus half sites are primarily intergenic within the C. crescentus genome.

A genome wide microarray analysis of the *Caulobacter* cell cycle revealed that approximately 150 RNA molecules are either directly or indirectly influenced by CtrA (18). However, of these genes, only 38 displayed the TTAA-N7-TTAA CtrA consensus sequences in the 5' upstream non-coding region of those genes (6). We therefore

analyzed the complete *C. crescentus* genome to address the frequency of CtrA TTAA motifs and their distribution within the chromosome (Figure 3). Assuming a random choice model, the statistical frequency of TTAA sequences within the *C. crescentus* genome (67% G-C content) predicts approximately 3000 TTAA sequences. However, the genome contains only 1200 sequences, suggesting this limitation has a regulatory significance. We narrowed our search to pairs of TTAA sequences separated by 0-20 nucleotides (Figure 3A). Of the 73 pairs identified, 66 (90%), mapped intergenically, a region that occupies less than 5% of the *Caulobacter* genome, and within 200 bp of 93 different open reading frames. Interestingly, this analysis also revealed that there are only 15 precise TTAA-N7-TTAA consensus-binding sites within the entire *C. crescentus* genome, four of which are conspicuously located within *Cori*. This suggests CtrA utilizes a separate class of binding sites to regulate the remaining promoters within its regulon. A further analysis of the *C. crescentus* genome reveals the *motB* promoter may also fall into this category because of the sequence similarity to the CtrA P1 and P2 promoters (Figure 3B). MotB ChIP profile shows a cell cycle pattern of binding similar to the *ctrA* promoter in both WT and CtrAΔ3 expressing cells. The pattern of binding to *motB* in WT cell is also consistent with the cell cycle regulation of *motB* (6).

Phosphorylation does not stimulate binding of CtrA to the P1 and P2 promoters.

We know that CtrA P1/P2 use CtrA *in vivo* (11), yet this DNA lacks TTAA-N7-TTAA organization. Instead P1/P2 have alternate groupings of half sites (Figure 4A) Since the *ctrA* promoters demonstrate an altered arrangement of CtrA binding sites, we wished to determine what role phosphorylation and co-operativity played in recruiting CtrA to this

promoter. A previous report demonstrated that CtrA bound to two distinct regions of the *ctrA* promoter (11). However, these experiments did not address how CtrA phosphorylation influenced binding to P1 and P2. We performed DNase I footprint assays to determine the binding affinities of phosphorylated (CtrA~P) and unphosphorylated CtrA to both promoters (Fig 4B). CtrA and CtrA~P bind P1 and P2 with low and equal affinities having a dissociation constant ($\mathbf{K_d}$) of ~300 nM. These results were unexpected since we have shown previously that CtrA phosphorylation increases binding to five sites within the origin of replication (16). Therefore, as a control, we performed parallel DNase I footprint experiments with the same CtrA~P preparations from Figure 3B. These footprints yielded the anticipated increase in DNA binding affinity of CtrA~P to sites a and b of *Cori* (Fig. 4C), but not to P1 and P2 of *ctrA*.

Abundance of cellular CtrA protein

The binding constants of the *ctrA* promoters suggest cellular abundance of CtrA must be high to favor binding. We carefully measured the copy number of CtrA molecules per cell in synchronous cultures by comparing immunoblots of *C. crescentus* cell lysates with those of purified CtrA protein. Figure 5outlines the methodology for quantitating CtrA in cells. Figure 7A shows a western blot titration of purified CtrA (2-20 ng) which was subsequently scanned and quantitated using Scion Imager software. The resulting densitometries were plotted and the slope determined from the linear region of the curve (Figure 5B). Figure 5C outlines a western blot of CtrA protein during the *C. crescentus* cell cycle. Densities from representative time points (t=0, t=120) were utilized for calculating the number of protein molecules per cell. Quantitation of cellular CtrA

reveals approximately 9000±1000 copies of CtrA/cell in swarmer (Figure 5C, t=0 min) and pre-divisional cells (Figure 5C, t=120 min) and supports previous findings (26). These data also demonstrate that *in vivo* CtrA concentrations are in excess of the predicted dissociation constant for CtrA binding to P1 and P2 (~0.3 μM) and suggests that in cells expressing CtrAΔ3, both promoters would be continuously occupied during the cell cycle without altering promoter activity.

Cell cycle occupancy of the ctrA promoter.

The phosphorylation-state-independent binding of CtrA~P to the *ctrA* promoter further suggests that occupancy may be the principle requirement for promoter regulation *in vivo* and that CtrA proteolysis alone would function to control these promoters. We analyzed occupancy of the *ctrA* promoters using a chromatin immunoprecipitation assay (Figure 6) which identifies protein-DNA interactions in growing cells that have been cross-linked with formaldehyde and lysed. Protein-DNA complexes are then immunoprecipitated with a specific antibody and the recovered material is gently heated to remove the crosslinks. The recovered DNA is then amplified by PCR and quantitated. Using synchronized *C. crescentus*, we investigated the fate of CtrA and CtrAΔ3 at the *ctrA* promoter (Figure 7A). Growing cells were sampled at t= 0 (swarmer), 45 (stalk), and 100 minutes (dividing cells) and immediately crosslinked with formaldehyde (see Methods). After recovery of DNA, samples were serially diluted and amplified with *ctrA* promoter-specific primers. Figure 7B and C provide a representative snapshot of the qualitative and quantitative data used to calculate the results in Figure 7A. Figure 7B demonstrate the agarose gel profile for undiluted and 1:8 diluted immunoprecipitated and mock

immunoprecipitated PCR reactions for each of the three time points measured. After scanning and measuring of gel signal densitometry, the slopes of the serial dilutions for mock and immunoprecipitated material were plotted (Figure 7C) and the ratio of the slopes reported (Figure 7A). For simplicity, the plot of t= 0 min (WT) was used for this example. Results indicate (Figure 6A) the occupancy of the *ctrA* promoter by CtrA is cell cycle regulated in wild type cells however in CtrAΔ3 expressing cells, CtrA occupies the *ctrA* promoter constitutively and we next investigate how constitutive binding to *ctrA* influences promoter activity.

A stable CtrA allele does not alter the cell cycle regulation of the P1 and P2 promoters. In figure 8A, the P1 and P2 promoters of *ctrA* were investigated separately and the affect of WT CtrA and CtrAΔ3 measured. Using synchronized *C. crescentus*, cells were pulse-labelled with S-35 and beta-galactosidase under control of either the P1 or P2 promoter were immunoprecipitated at various time points and the relative radioactive intensities plotted. Western blots of CtrA and CtrAΔ3 were used to track protein abundance during the cell cycle (Figure 8B). In Figure 8A (left), the P1 promoter demonstrates maximal activity at approximately 50 minutes corresponding to an absence of CtrA protein (Figure 8B top panel). However, upon the reemergence of CtrA (60 min), the P1 promoter is rapidly suppressed. In CtrAΔ3 expressing cells, CtrA is constitutively expressed however the timing of P1 is not greatly influenced however the transactivating potential is reduced by approximately 25%. P2 activity (Figure 8A, right) is stimulated later than P1 with maximal activity around 150 min followed by rapid suppression prior to cell division. Once again the temporal control of P2 in WT and CtrAΔ3 expressing cells is not

influenced although the maximal output of P2 in CtrAΔ3 cells is reduced by ~20%. The reduction of promoter activity in CtrAΔ3 expressing cells may be a result of the over-abundance of CtrAΔ3 in these cells. In our experimental system, CtrAΔ3 is under control of the heterologous xylX promoter and non-physiological expression levels of the transgene are achieved in the presence of xylose (data not shown). In order to limit unwanted synthesis of CtrAΔ3, cells were grown in glucose to achieve basal level expression from this promoter.

CtrA binds upstream of motB in vivo. In an attempt to identify other promoter regions that might have similar structure to the CtrA promoter, a sequence analysis of the *Caulobacter* genome revealed a region, upstream of *motB*, similar in respect to the *ctrA* promoter (Figure 9A). The striking homology of the putative promoter region of *motB* suggests it may also interact with CtrA in a cell cycle dependent fashion. This region showed a selective interaction with both CtrA and CtrAΔ3, evidence that may suggest *motB* is under CtrA regulation. In a previous study which measured the global transcription patterns of 3000 open reading frames in the *Caulobacter* genome, *motB* demonstrated a cell cycle pattern of transcription (18). In a transcriptional analysis of the CtrA regulon (6) *motB* was not identified as a target of CtrA regulation. However in this study nearly 20% of the putative promoter regions that contained DNA consensus sequences for CtrA were also not identified.

DISCUSSION

The response regulator CtrA is part of a transcriptional hierarchy that regulates the expression of numerous genes including those responsible for flagellar biosynthesis and assembly (2). The major genetic determinant for CtrA regulation is the organization of CtrA consensus sequences TTAA-N7-TTAA. Analysis of TTAA distribution indicates an intergenic bias, most favorably within 5' upstream non coding region of many genes (Figure 3).

The ctrA Promoter

It has been previously demonstrated that CtrA~P has increased affinity for five consensus binding sites within the *Caulobacter* origin of replication (16). However, while a few promoters contain perfect consensus sequences such as the *ftsZ* promoter (27) many of the putative promoter regions of CtrA-regulated genes have either degenerate consensus sequences or binding sites of varying length (N≠7). One example is the *fliX* promoter which houses only a single TTAA half site yet appears sufficient for binding and transactivation by CtrA (28). Also, the *che* promoter which controls the expression of the major chemotaxis operon as well as a novel gene *cagA* lack good CtrA binding sites but are directly dependent on CtrA for expression (29).

CtrA expression is autoregulated through its binding to two promoters (11). The pattern of binding and the role of *ctrA* regulation are markedly different within these two promoters. It has been demonstrated that binding of CtrA to P1 represses transcription of *ctrA*. However, new evidence suggests the methylation state of P1 (30) and the binding of

the newly identified response regulator GcrA (31) play an important role in controlling the activity of this promoter *in vivo* . In contrast to P1, CtrA binding to P2 stimulates transcription prior to cell division. Our DNaseI footprint results demonstrated that CtrA phosphorylation did not increase binding for P1 or P2 *in vitro*. These findings support previous reports that phosphorylation does not enhance binding to DNA by PhoB a member of the OmpR/PhoB subfamily but rather strengthens protein-protein interactions with the sigma70 subunit of RNA polymerase (32). We also believe that phosphorylation of CtrA plays an allosteric role in recruiting RNA polymerase to the *ctrA* promoters either through changes in DNA topology or stability of protein-protein interactions. The *in vitro* Kd for CtrA and CtrA~P at the *ctrA* promoter is poor (~300nM) and the high cellular abundance of CtrA would favor binding to P1 and P2 *in vivo*. In swarmer cells, when CtrA is most abundant (~10^4 copies/cell) it is also phosphorylated and active (33). Paradoxically, the P2 promoter, which is activated by CtrA, is selectively repressed in swarmer cells and suggests that this promoter is regulated differently in swarmer cells.

It has been previously suggested that derepression of the P1 promoter is achieved through proteolytic turnover of CtrA during stalk cell differentiation (2). However, in our experiments, proteolysis is dispensable and cells expressing stable CtrAΔ3 did not alter cell cycle timing of the P1 promoter. Our data is supported by previous observations that stabilization of CtrA, in a $DivK^{TS}$ mutant grown at non permissive temperature, also fails to alter the temporal regulation of the *ctrA* promoter (34).

The work presented here along with our previous work (16) identify at least three modes of CtrA binding (Figure 10). The first mode encompasses CtrA binding sites **a** and **b** in *Cori* whereby CtrA phosphorylation stimulates co-operativity between adjacent sites. The second mode, which defines most response regulators, is increased affinity for DNA upon phosphorylation such as binding sites **a-e** in *Cori* including the flagellar and *ccrM* promoters (35,36). The third mode and the one revealed here does not stimulate CtrA binding upon phosphorylation and may be restricted in part to promoters having atypical binding sites such as the *ctrA* promoter. These three modes of CtrA binding reveals that simple recruitment by phosphorylation is too limited a view for understanding how CtrA functions at specific DNA loci. Our data suggest the static occupation of CtrA within a promoter is not sufficient for regulation of transcription and that phosphorylation provides an allosteric mechanism for communicating with other components of the promoter complex such as the RNA polymerase holoenzyme.

A New Model of ctrA-regulated Gene Transcription

We demonstrate and confirm (26) that the abundance of CtrA greatly exceeds the binding constants for weak CtrA binding sites (i.e. the *ctrA* promoter) and that CtrA constitutively occupies these sites when over expressed without disrupting temporal gene expression. Previous work has shown that a constitutively active CtrA allele (CtrAD51E) failed to demonstrate increased binding to *Cori in vitro* yet provided all necessary *in vivo* functions when expressed in a CtrA null mutant (5). This view of CtrA regulation suggests the increased affinity of phosphorylated CtrA for target DNA may be dispensable. These data suggest phosphorylation provides distinct biochemical properties extending beyond

the mere requisite for DNA binding. The standard transcriptional model states that phosphorylation of transactivators is the primary signal for focusing biological activity because phosphorylation shifts the equilibrium towards the bound state of the protein for its target sequence(s). The model presents a static view of transcription and suggests transcription signals are based on the role of transcription factors as spatial markers. Our data presents an allosteric model of transcription (Figure 11) and suggest that occupancy alone is not sufficient to repress P1 transcription, and likewise, it is not sufficient to activity transcription at P2. Under this model, phosphorylation permits dynamic communication (new surface contacts) with other components of the transactivation complex to promote or repress transcription. In *Cori*, CtrA is a repressor of transcription and replication (1) and suggests a similar allosteric model may functions at *Cori.* If this is correct, CtrA phosphorylation may be necessary to permit the recruitment of other origin binding proteins necessary to control transcription and replication within this region of the chromosome.

FIGURES AND TABLES

Table 2. Bacterial strains and plasmids

Strain/plamid	Genotype or description	Reference
E.coli		
BL21	F minus, *lon, ompT, hsdS$_B$*	
S17-1	E.coli 294::RP4-2(Tc::Mu) (Km::Tn7)	
C.crescentus		
NA1000	wild type *Caulobacter crescentus*	
Plasmids		
pTrc7.4	His6 N-terminal tagged CtrA protein	Quon et al. (1996)
pKJH5	MBP-EnvZ kinase protein fusion	Huang and Igo (1996)
pRK290lacZ	RK2-based lacZ transcription reporter	Gober and Shapiro (1992)
pLS2747	CtrAΔ3 fused to the xylose promoter	Domian et al (1997)
pLS3146	pRK290lacZ::ctrA promoter P1	Domian et al (1999)
pLS3147	pRK290lacZ::ctrA promoter P2	Domian et al (1999)
pBluescript II	SK(+) cloning vector	
pGM1877	Cori HindIII at +214 to XhoI in pSK(+)	

Figure 1. The *Caulobacter* cell cycle. The cycle begins with a non-replicating chemotactic swarmer cell (Sw) which differentiates to the replicating stalk cell (St). Growth of the pre-divisional cell produces a new flagellated swarmer pole. Segregating chromosomes are positioned in both the non-replicating swarmer (rep-) and the replication competent stalk cell (rep+). Shading indicates the temporal and spatial presence of the CtrA response regulator.

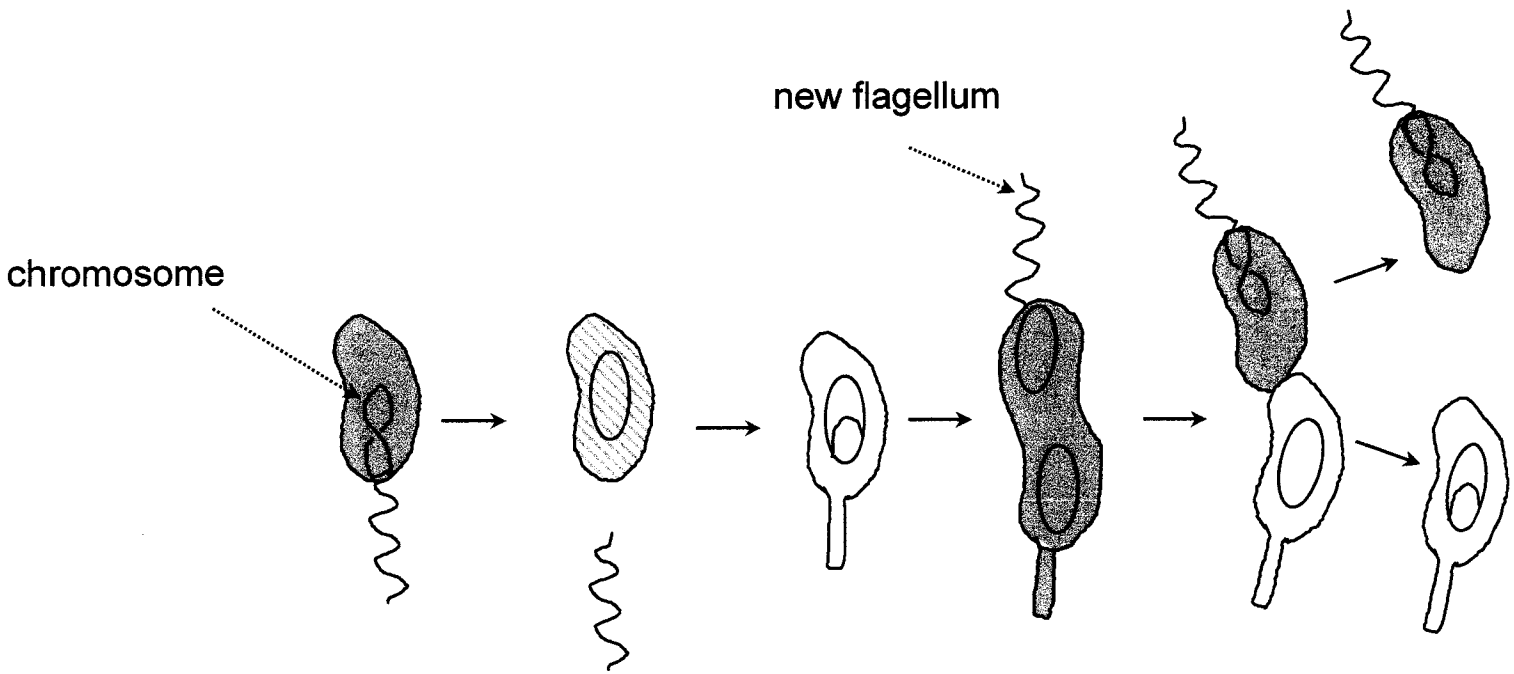
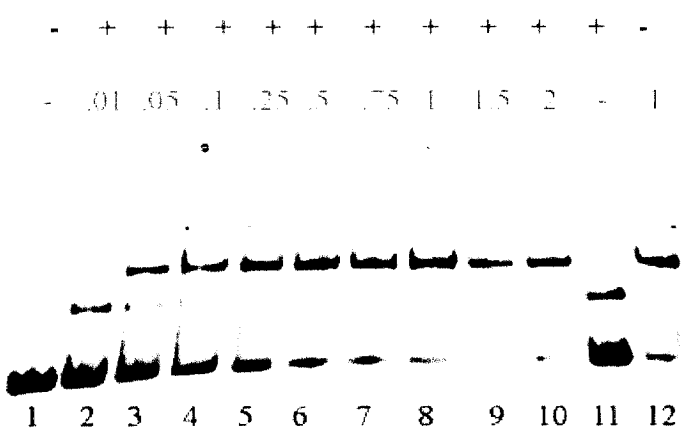

new flagellum
chromosome

Figure 2. *Electromobility shift assays using a CtrA-regulated promoter.* A) Diagramatic representation of expected electromobility gel shift of a His6-CtrA and GST-CtrA dimer *in vitro*. His6-CtrA homo-dimers (first column) and GST-CtrA homo-dimers (second column) have significantly different molecular weights and show distinct electromobilities. Combinations of His6-CtrA and GST-CtrA should produce hetero-dimers with an intermediate electromobility and easily distinguished from the two homo-dimers (third column). B) Electromobility shift assays of His6-CtrA/GST-CtrA mixtures. Using a fixed concentration of His6-CtrA (1 μg/ml) increasing concentrations of GST-CtrA (μg/ml) were added (lanes 2-10). Lane 1 represents a no protein control including unmixed His6-CtrA (lane 11) GST-CtrA (lane 12). C) Comparison of His6-CtrA and GST-CtrA binding to a test promoter in which the leftward TTAA has been disrupted (mutant promoter). Lanes 7 and 8 showing the electrobility shift for His6-CtrA and GST-CtrA binding to the wild-type promoter.

A


GST-CtrA Dimers
GST-CtrA/His6-CtrA Dimer
His6-CtrA Dimers
Free Probe

B


- + + + + + + + + + + -
His6-CtrA
- .01 .05 .1 .25 .5 .75 1 1.5 2 - 1
GST-CtrA
1 2 3 4 5 6 7 8 9 10 11 12

C

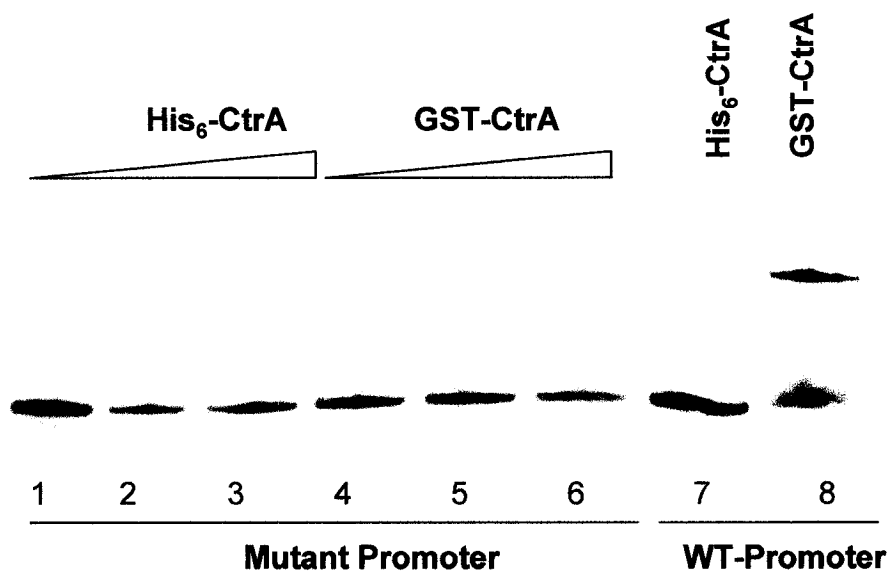

His6-CtrA
GST-CtrA
His6-CtrA
GST-CtrA
1 2 3 4 5 6 7 8
Mutant Promoter
WT-Promoter

Figure 3. *Genomic distribution of putative CtrA binding sites.* A). The relative spacing (1-20 nt) distribution of 73 pairs of CtrA half site motifs (TTAA) identified in the *Caulobacter* genome. The quantity of each group is shown (shaded bars) alongside the intergenic frequency (open bars) within the genome.

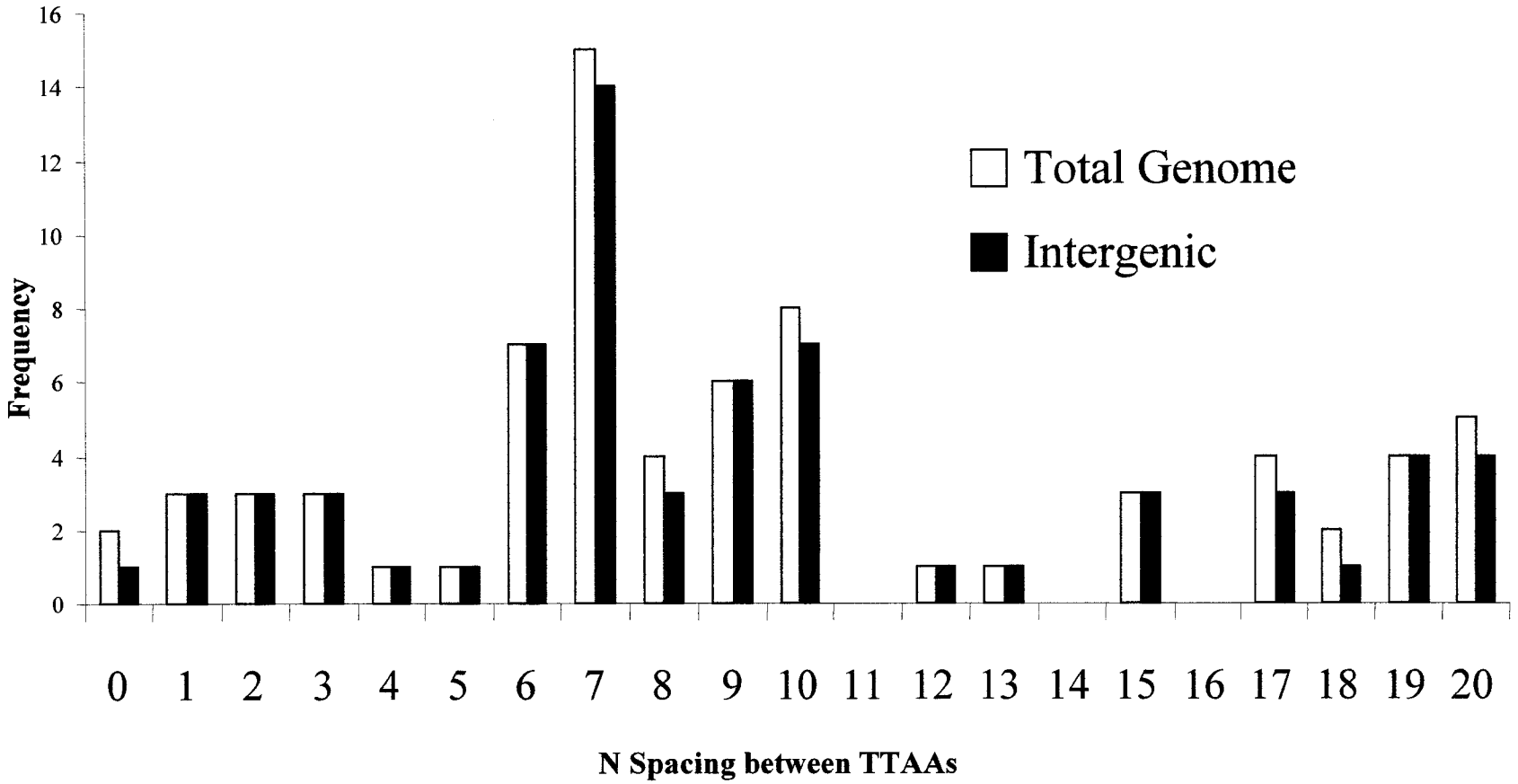

16
14
12
10
8
6
4
2
0
Frequency
Total Genome
Intergenic
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
N Spacing between TTAAs

Figure 4. *Dnase I Footprint of C. crescentus ctrA promoters P1 and P2.* (A) Schematic representation of the P1 and P2 promoters of ctrA. Boxes indicate TTAA half sites and their relative orientation within the promoter region. Reporter constructs were designed as outlined in material and methods. (B) α^{32}P end labeled XmnI fragment of the *ctrA* promoter (20,000 CPM) was incubated with increasing concentrations of phosphorylated and unphosphorylated wild type CtrA protein (0.2-1 uM). (C) DnaseI footprint control experiment of Cori using the same phosphorylated CtrA preparation.

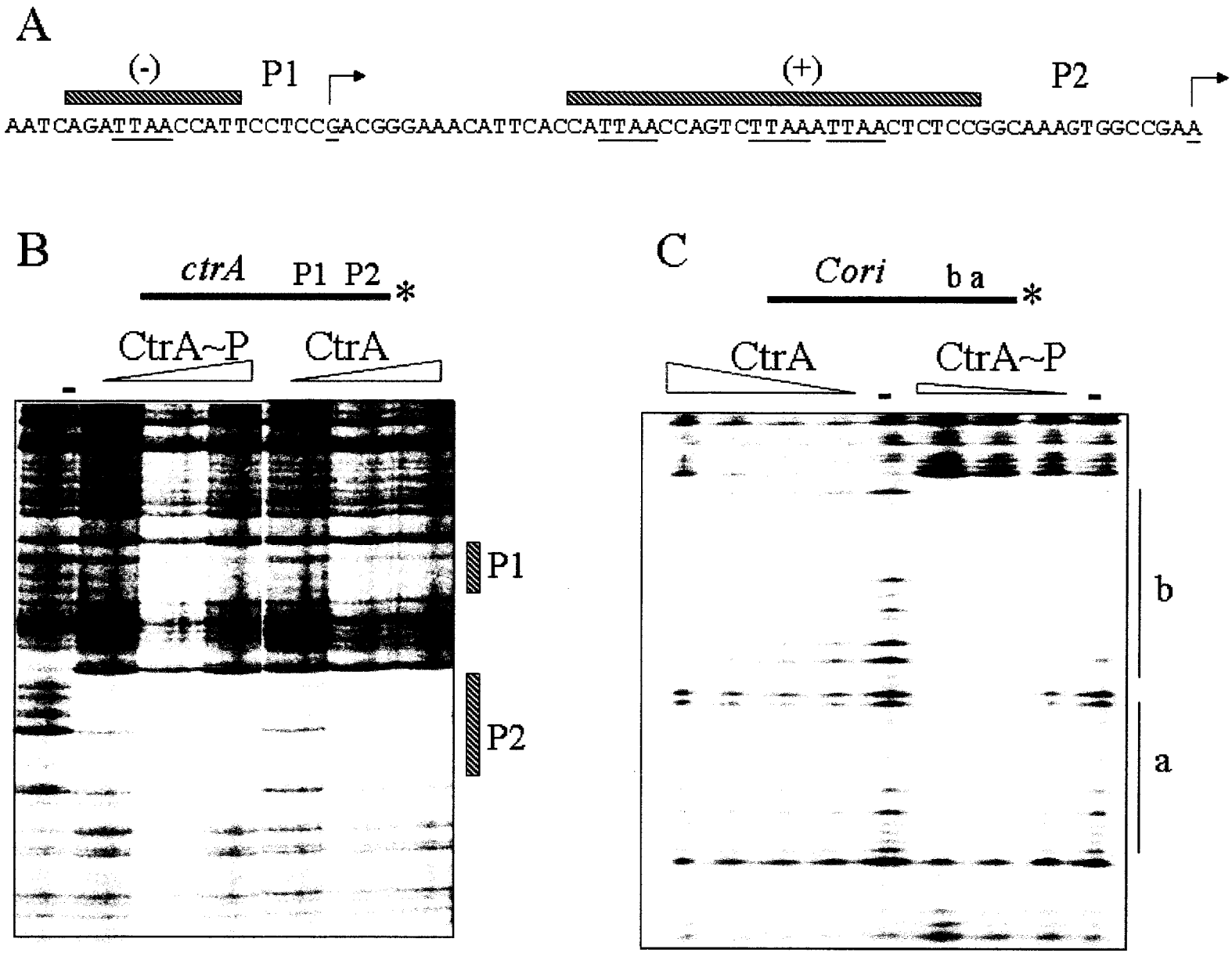
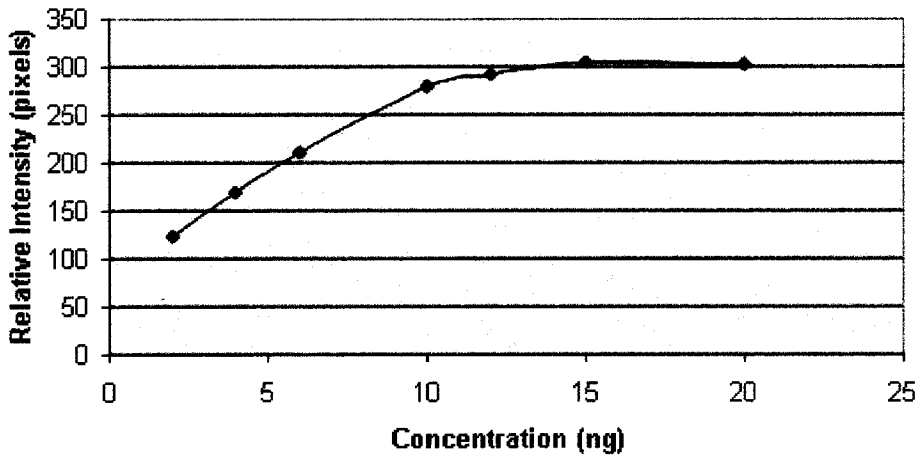

A
(-)
P1
(+)
P2
AATCAGATTAACCATTCCTCCGACGGGAAACATTCACCATTAACCAGTCTTAAATTAACTCTCCGGCAAAGTGGCCGAA
B
ctrA
P1 P2
*
CtrA~P
CtrA
P1
P2
C
Cori
b a
*
CtrA
CtrA~P
b
a

Figure 5. *Quantitation of CtrA protein in cells.* A) Western blot of purified CtrA protein (2-20ng) B) Signal intensity for purified CtrA was determined by densitometric analysis and plotted and the slope of the line calculated. C) Representative western blot of CtrA cell cycle regulation in synchronized *Caulobacter* cells. Data from three separate experiments were pooled and the number of CtrA protein molecules per cell was determined at t=0 and t=120 (see results)

A

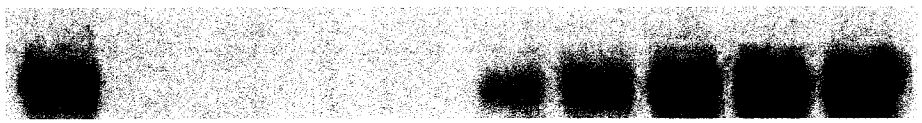
2 4 6 10 12 15 20 (ng)

B



C

0 15 30 45 60 75 90 120 150 180 (min)



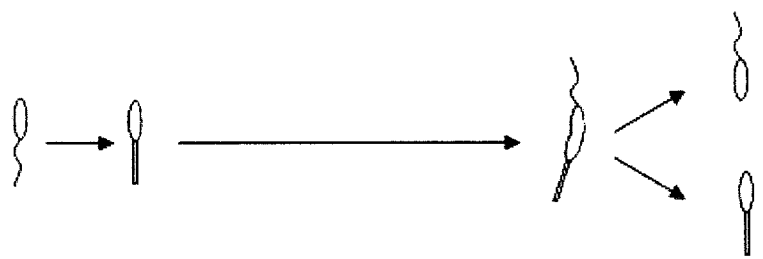


Figure 6. *Schematic of the Chromatin ImmunoPrecipitation Assay (ChIP).* Exponentially growing cells are treated with formaldehyde to cross protein and DNA in vivo followed by cell lysis and sonication to reduce the genomic DNA into fragments of 500-1000 bp. The cross-linked DNA is immunoprecipitated with the appropriate antibody and a mock IP (no antibody) is run in parallel as a negative control. Bound antibodies are recovered by protein A sepharose separation and washed extensively with immunoprecipitation buffer and finally resuspended in TE. Cross-links are reversed overnight at 65°C the resulting supernatant is preserved for competitive PCR.

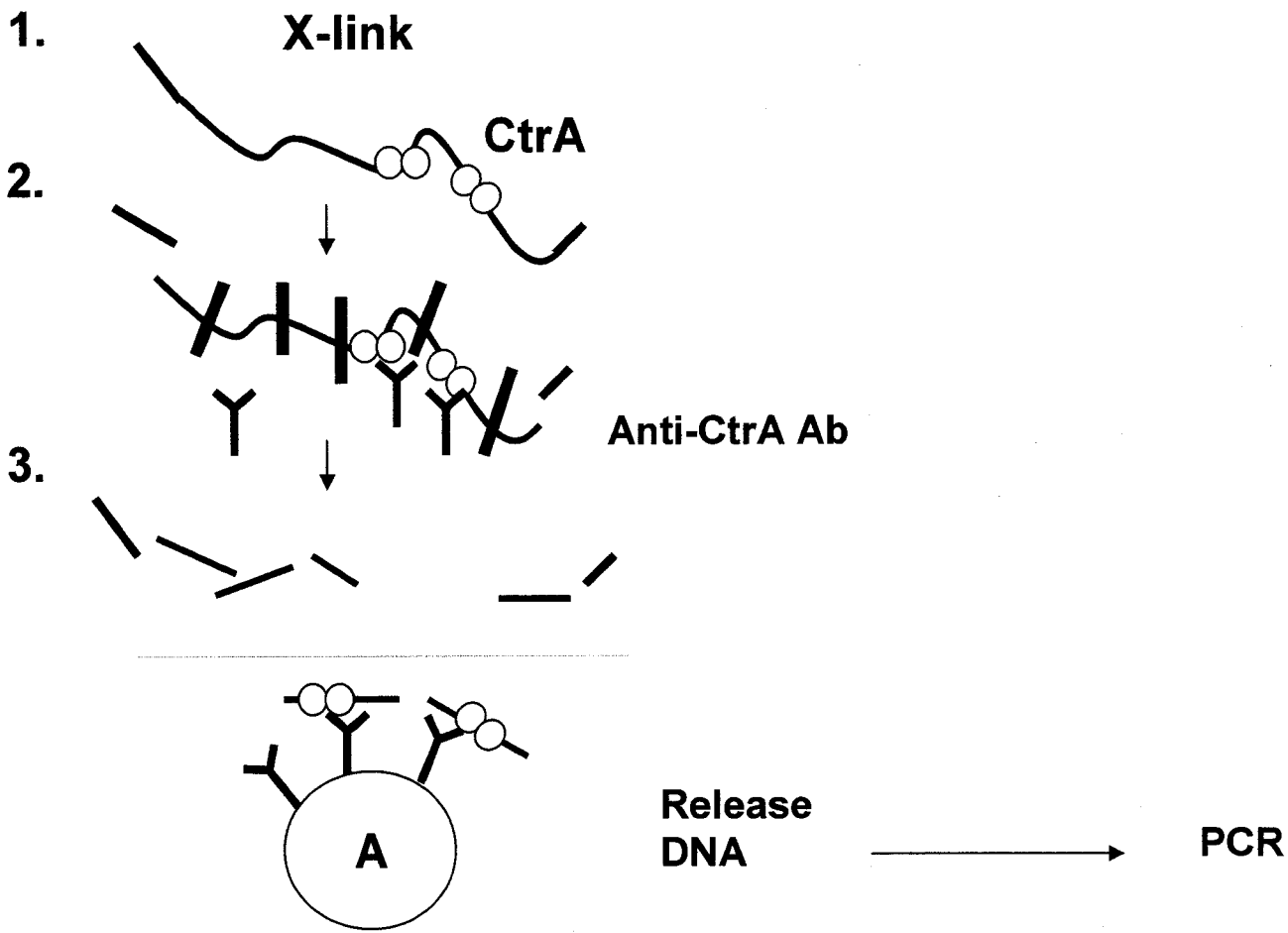
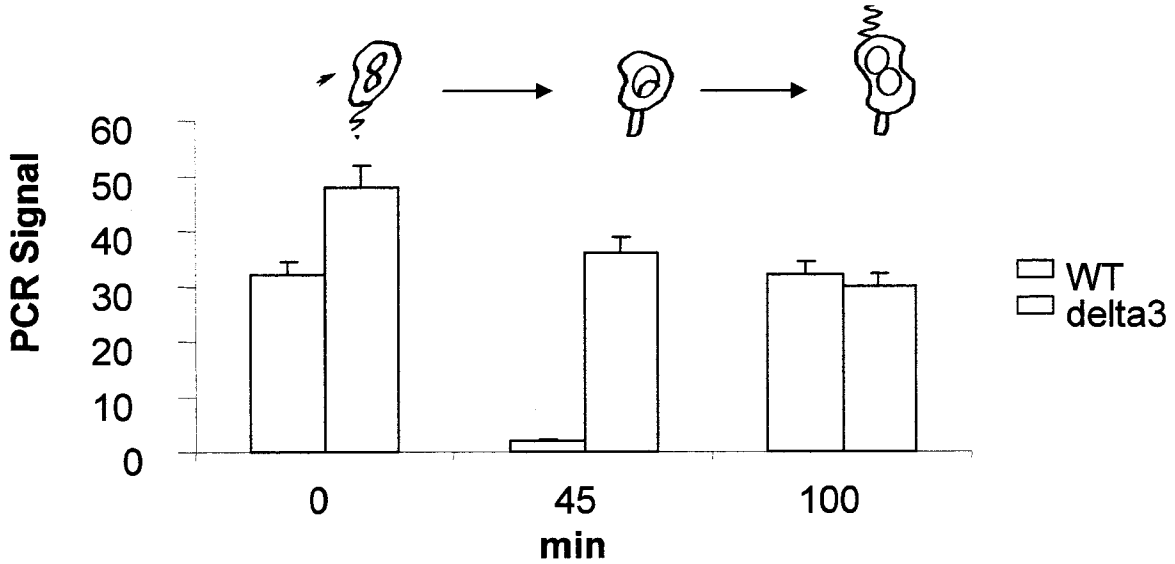
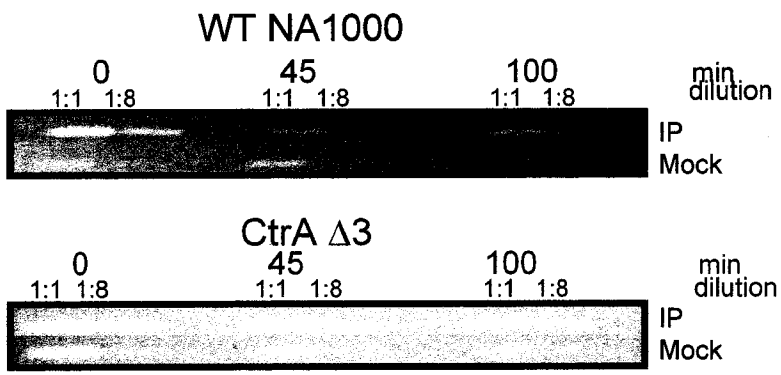

1.
X-link
CtrA
2.
Anti-CtrA Ab
3.
A
Release
DNA
PCR

Figure 7. *Chromatin immunoprecipitation of the ctrA promoter in synchronized Caulobacter cells.* Synchronized cell populations of *Caulobacter* expressing either WT CtrA or CtrAΔ3 were fixed by formaldehyde and protein crosslinked genomic DNA was immunoprecipitated with CtrA antisera. A) Histogram demonstrates the ratio of slopes for mock and immunoprecipitated PCR titration curves calculated from 5C. B) Photograph images comparing the qualitative intensities of mock and immunoprecipitated PCR reactions for CtrA and CtrAD3 expressing cells at t=0, 45, and 100 min. Each time point shows a representative comparison of undiluted and 1:8 diluted signals. C) Densitometry of each titration point in each PCR reaction was quantitated and plotted as a function of pixel density. A representative plot of t=0 for WT cells is shown.

A


PCR Signal
60
50
40
30
20
10
0
0
45
100
min
WT
delta3

B


WT NA1000
0
45
100
min
1:1 1:8
1:1 1:8
1:1 1:8
dilution
IP
Mock
CtrA Δ3
0
45
100
min
1:1 1:8
1:1 1:8
1:1 1:8
dilution
IP
Mock

C

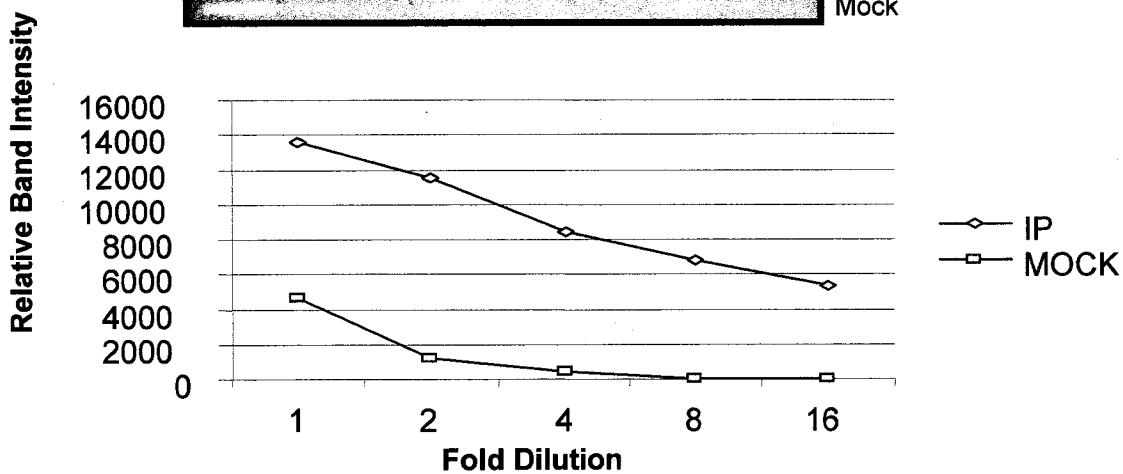

Relative Band Intensity
16000
14000
12000
10000
8000
6000
4000
2000
0
1
2
4
8
16
Fold Dilution
IP
MOCK

Figure 8. *Cell cycle regulation of the ctrA promoter.* (A) Independent expression profiles for the P1 and P2 ctrA promoters in synchronous *Caulobacter* preparations. Beta-galactosidase was immunoprecipitated from cells pulse-labeled with ^{35}S-methionine and quantitated by phosphor-imaging. Transactivation profiles of the P1 promoter (left panel) and the P2 promoter (right panel) in wild-type *Caulobacter* cells expressing either endogenous or proteolytically stable CtrAΔ3. Units are expressed as relative signal intensity in comparison to WT cells (B) Western blot analysis of CtrA (top panel) and CtrAΔ3 (bottom panel) expression in synchronized cells.

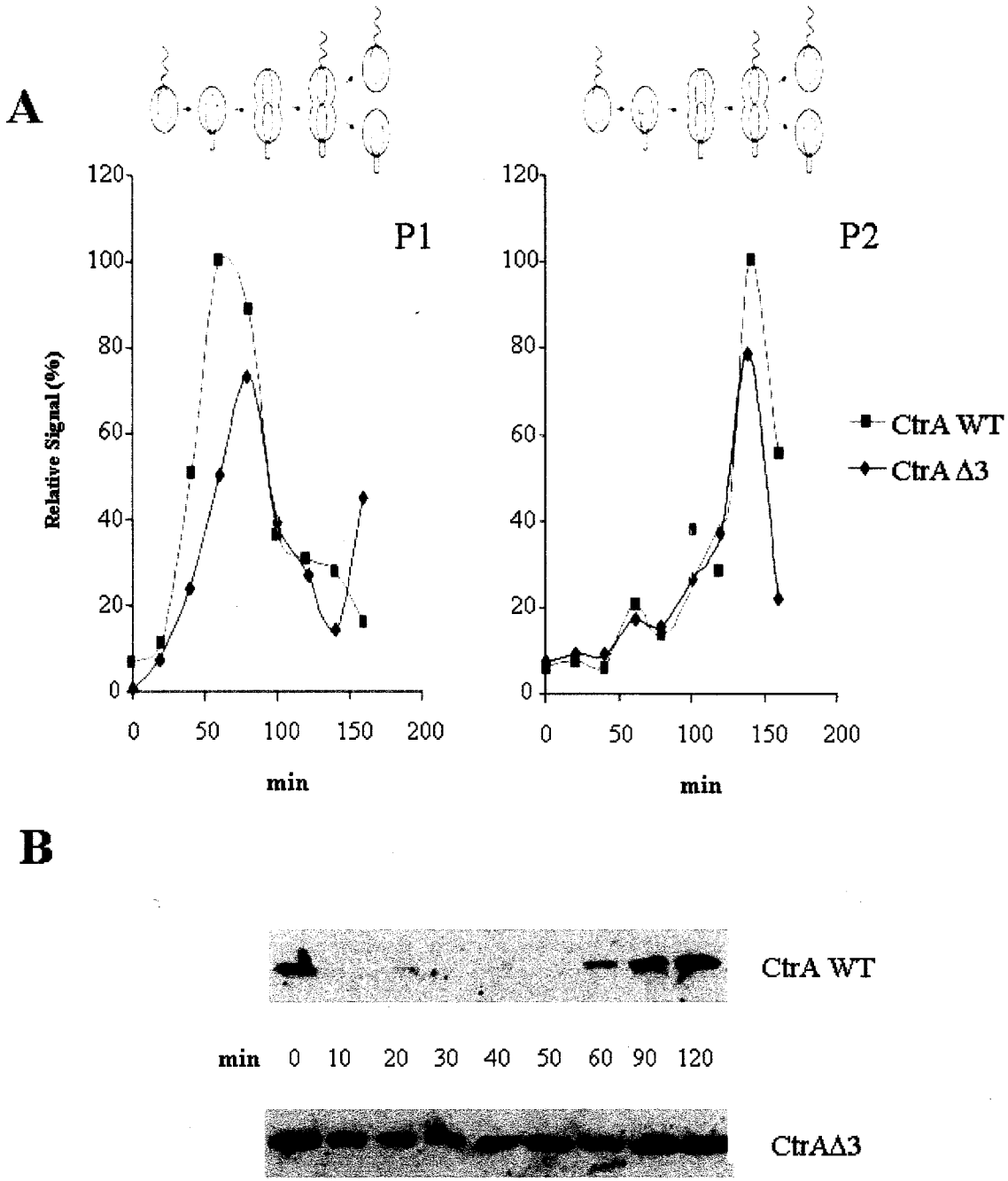
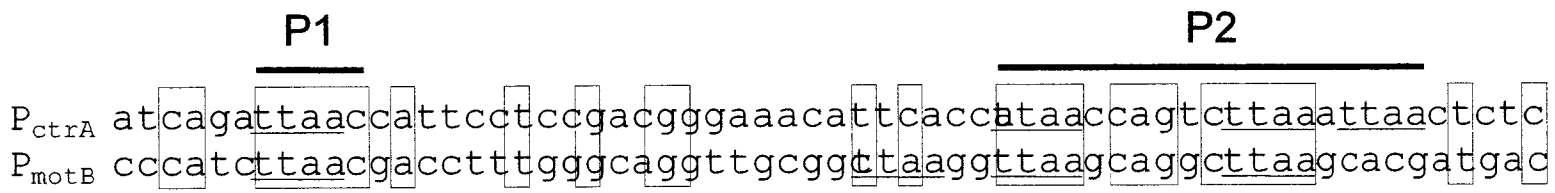

A
P1
P2
Relative Signal (%)
120
100
80
60
40
20
0
0
50
100
150
200
min
CtrA WT
CtrA Δ3
B
CtrA WT
min 0 10 20 30 40 50 60 90 120
CtrAΔ3

Figure 9. *CtrA binds upstream of motB in vivo.* Similar to Figure 5, CtrA chromatin immunoprecipitation experiments were performed and the upstream (promoter) region of *motB* was analyzed by PCR in WT cells or cells expressing CtrAD3. As for Figure 5, histograms were calculated from titration curves for mock and immunoprecipitated samples and signal (fold over mock) is equal to the ratio of the slopes from each curve.

A


P1
P2
P_{ctrA} atcagattaaccattcctccgacgggaaacattcacctataaccagtcttaaattaactctc
P_{motB} cccatcttaacgacctttgggcaggttgcggcttaaggttaagcaggcttaagcacgatgac

B

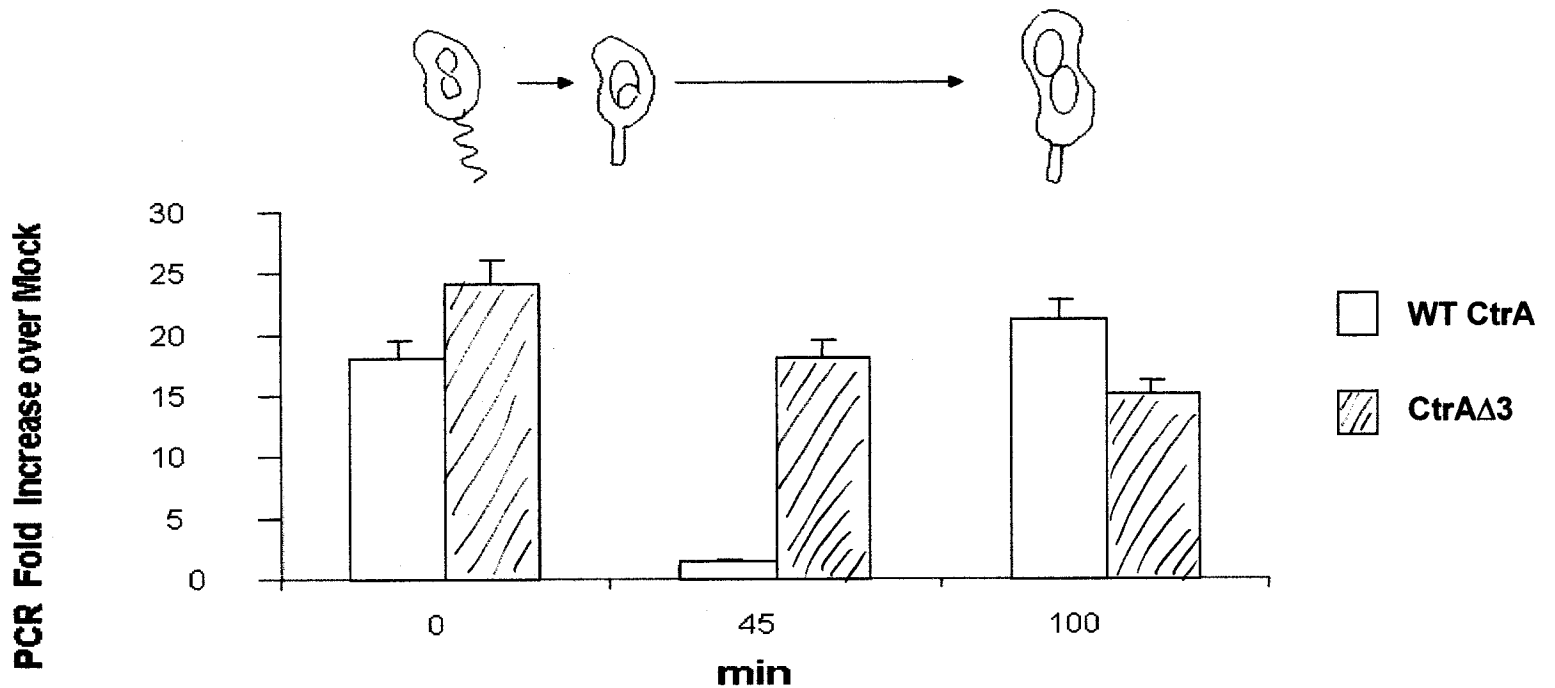

PCR Fold Increase over Mock
30
25
20
15
10
5
0
0
45
100
min
WT CtrA
CtrAΔ3

Figure 10. *CtrA demonstrates three modes of DNA binding.* (A) The modes of CtrA binding are outlined based on this work and previous findings. 1) CtrA phosphorylation stimulates increased affinity for DNA (binding sites a-e in *Cori*). 2) CtrA phosphorylation stimulates cooperativity between adjacent binding sites (binding sites a-b in *Cori*). 3) Phosphorylation does not stimulate binding to the *ctrA*, and possibly other, promoters (this work).

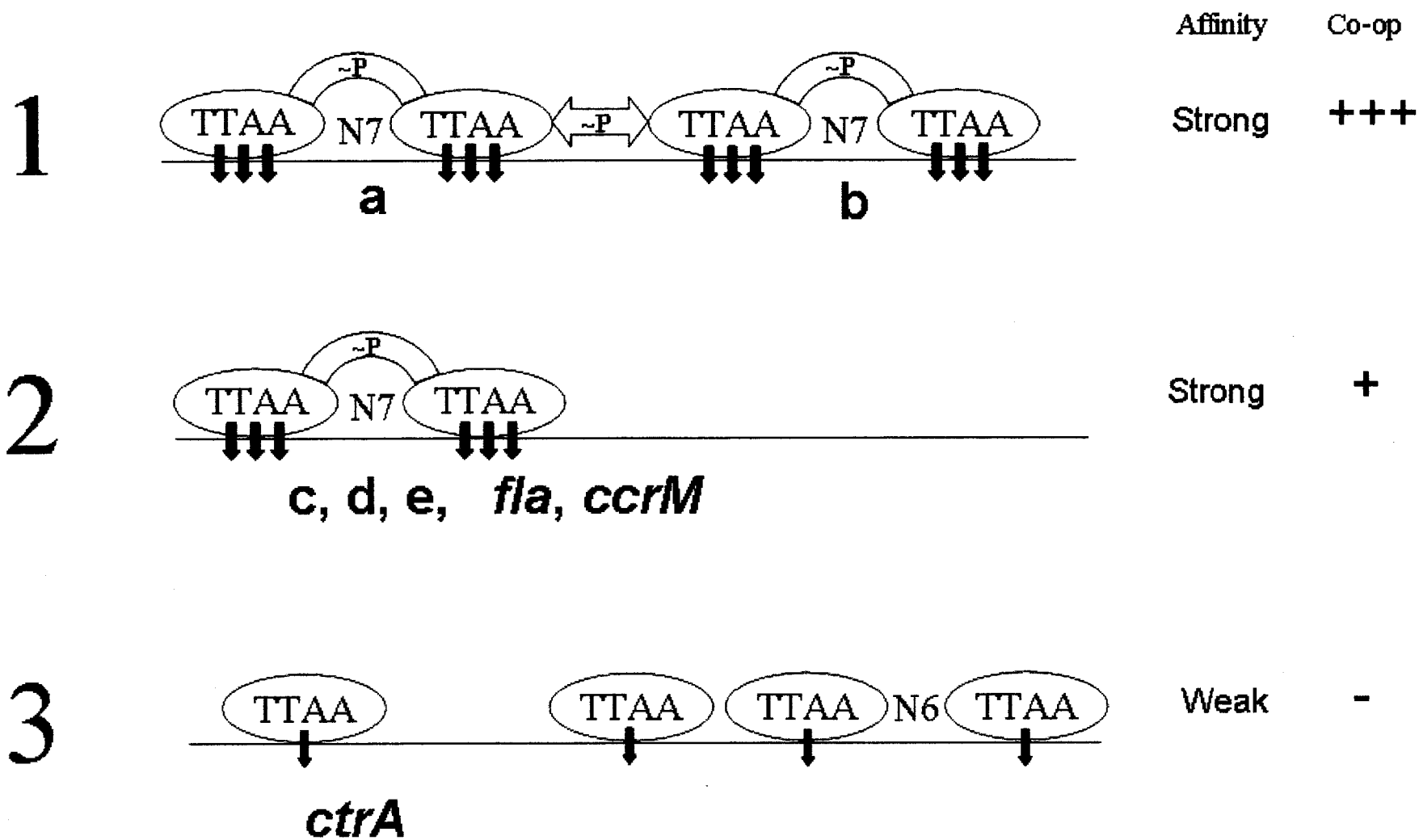
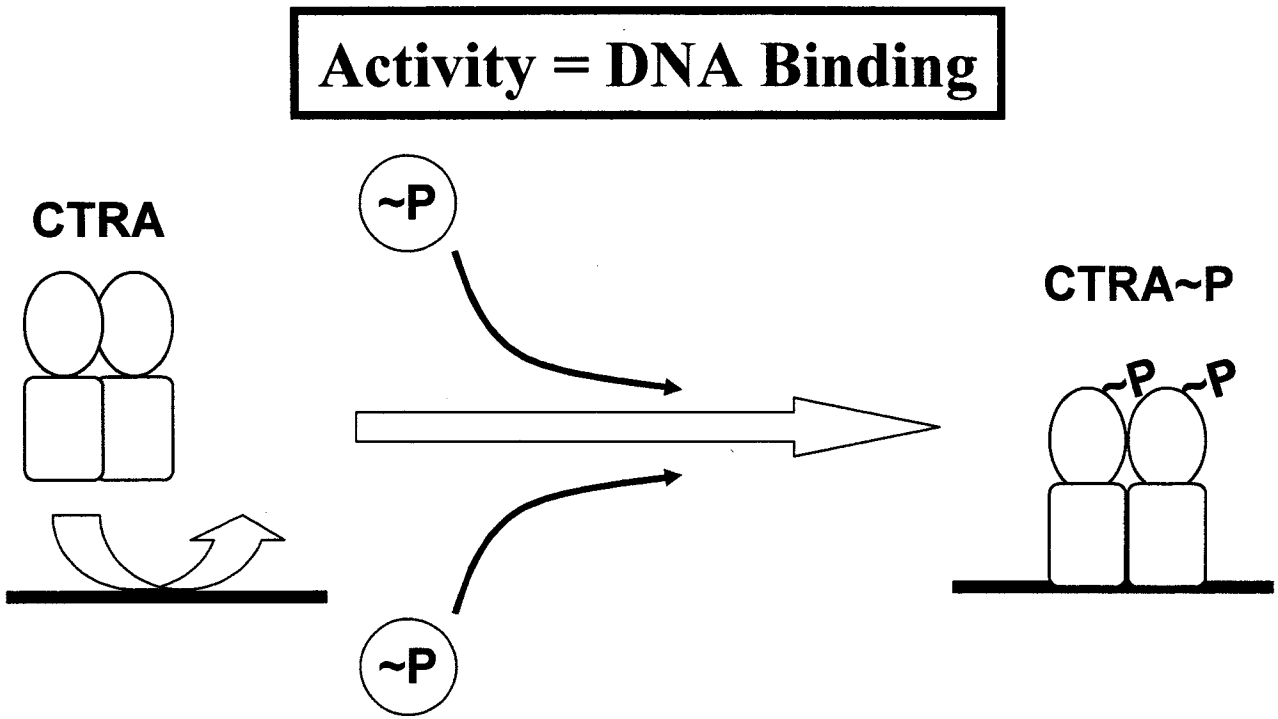
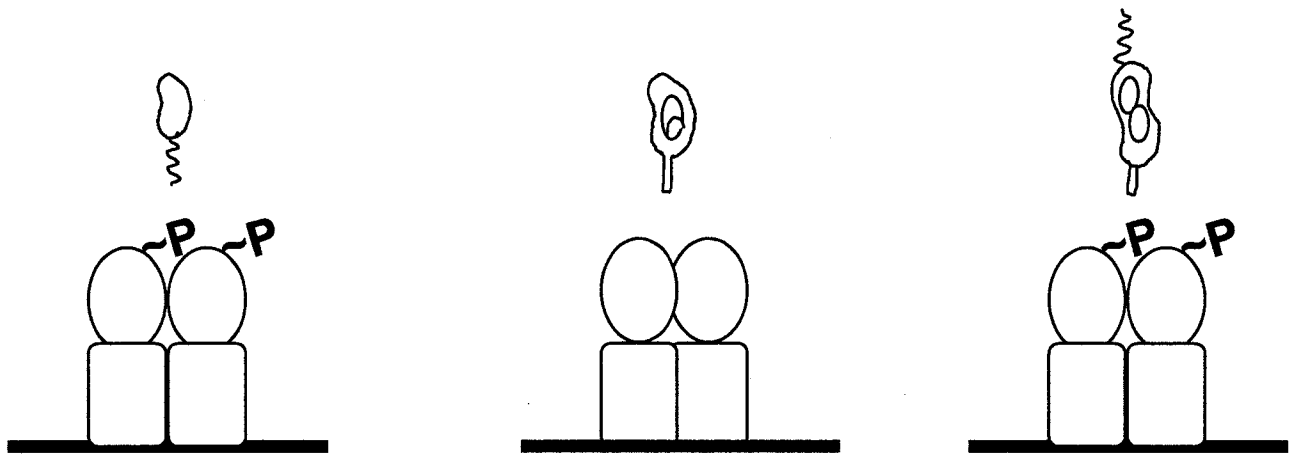

Affinity
Co-op
1
~P
TTAA
N7
TTAA
~P
TTAA
N7
TTAA
Strong
+++
a
b
2
~P
TTAA
N7
TTAA
Strong
+
c, d, e, fla, ccrM
3
TTAA
TTAA
TTAA
N6
TTAA
Weak
-
ctrA

Figure 11. *Allosteric model of CtrA-regulated transcription.* Occupancy of CtrA binding sites by CtrA is not the minimal signal for promoter regulation a feature which has been previously demonstrated for NR_I a regulatory component of nitrogen assimilation in *E. coli* (37). A) Unphosphorylated CtrA is not capable of forming stable complexes with DNA (left) and therefore is unlikely to stimulate transcription. Only upon phosphorylation (right) can CtrA interact with target DNA and stimulate transcription. This suggests that activity of the *ctrA* promoter is dependent on the DNA binding of CtrA. B) Continuous occupation of CtrA at the *ctrA* promoter is tolerated and does not disrupt the cell cycle regulation of this promoter (this work). The model proposes 1) that phosphorylation provides new surface contacts necessary for the stimulation of transcription and 2) that binding to DNA in the absence of phosphorylation does not stimulate transcription.

A



B



Activity = New surface contacts

References

1. Marczynski, G.T. and Shapiro, L. (2002) Control of chromosome replication in *Caulobacter crescentus*. *Annual Reviews in Microbiology*, **56**, 625-656.

2. Quon, K.C., Marczynski, G.T. and Shapiro, L. (1996) Cell cycle control by an essential bacterial two-component signal transduction protein. *Cell*, **84**, 83-93.

3. Jenal, U. and Fuchs, T. (1998) An essential protease involved in bacterial cell cycle control. *The EMBO Journal*, **17**, 5658-5669.

4. Domian, I.J., Quon, K.C. and Shapiro, L. (1997) Cell type-specific phosphorylation and proteolysis of a transcriptional regulator controls the G1 to S transition in a bacterial cell cycle. *Cell*, **90**, 415-424.

5. Siam, R. and Marczynski, G.T. (2003) Glutamate at the phosphorylation site of response regulator CtrA provides essential activities without increasing DNA binding. *Nucleic Acids Research*, **31**, 1775-1779.

6. Laub, M.T., Chen, S.L., Shapiro, L. and McAdams, H.H. (2002) Genes directly controlled by CtrA, a master regulator of the Caulobacter cell cycle. *Proc Natl Acad Sci U S A*, **99**, 4632-4637.

7. Barnett, M.J., Hung, D.Y., Reisenauer, A., Shapiro, L. and Long, S.R. (2001) A homolog of the CtrA cell cycle regulator is present and essential in Sinorhizobium meliloti. *J Bacteriol*, **183**, 3204-3210.

8. Brassinga, A.K., Siam, R., McSween, W., Winkler, H., Wood, D. and Marczynski, G.T. (2002) Conserved response regulator CtrA and IHF binding

sites in the alpha-proteobacteria Caulobacter crescentus and Rickettsia prowazekii chromosomal replication origins. *J Bacteriol*, **184**, 5789-5799.

9. Dasgupta, N., Ferrell, E.P., Kanack, K.J., West, S.E. and Ramphal, R. (2002) fleQ, the gene encoding the major flagellar regulator of Pseudomonas aeruginosa, is sigma70 dependent and is downregulated by Vfr, a homolog of Escherichia coli cyclic AMP receptor protein. *J Bacteriol*, **184**, 5240-5250.

10. Kahng, L.S. and Shapiro, L. (2001) The CcrM DNA methyltransferase of Agrobacterium tumefaciens is essential, and its activity is cell cycle regulated. *J Bacteriol*, **183**, 3065-3075.

11. Domian, I.J., Reisenauer, A. and Shapiro, L. (1999) Feedback control of a master bacterial cell cycle regulator. *Proc. Natl. Acad. Sci. USA*, **96**, 6648-6653.

12. Jacobs, C., Domian, I.J., Maddock, J.R. and Shapiro, L. (1999) Cell cycle-dependent polar localization of an essential bacterial histidine kinase that controls DNA replication and cell division. *Cell*, **97**, 111-120.

13. Sciochetti, S.A., Lane, T., Ohta, N. and Newton, A. (2002) Protein sequences and cellular factors required for polar localization of a histidine kinase in Caulobacter crescentus. *J Bacteriol*, **184**, 6037-6049.

14. Lam, H., Matroule, J.Y. and Jacobs-Wagner, C. (2003) The asymmetric spatial distribution of bacterial signal transduction proteins coordinates cell cycle events. *Dev Cell*, **5**, 149-159.

15. Ryan, K.R., Huntwork, S. and Shapiro, L. (2004) Recruitment of a cytoplasmic response regulator to the cell pole is linked to its cell cycle- regulated proteolysis. *Proc Natl Acad Sci U S A*, **101**, 7415-7420.

16. Siam, R. and Marczynski, G.T. (2000) Cell cycle regulator phosphorylation stimulates two distinct modes of binding at a chromosome replication origin. *The EMBO Journal*, **19**, 1138-1147.

17. Quon, K.C., Yang, B., Domian, I.J., Shapiro, L. and Marczynski, G.T. (1998) Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin. *Proc. Natl. Acad. Sci. USA*, **95**, 120-125.

18. Laub, M.T., McAdams, H.H., Fraser, C.M. and Shapiro, L. (2000) Global analysis of the genetic network controlling a bacterial cell cycle. *Science*, **290**, 2144-2148.

19. Mohr, C., MacKichan, J.K. and Shapiro, L. (1998) A Membrane-Associated Protein, FliX, Is required for an Early Step in *Caulobacter* Flagellar Assembly. *Journal of Bacteriology*, **180**, 2175-2185.

20. Simon, R., Priefer, U. and Puler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: Transposon mutagenesis in Gram negative bacteria. *Biotechnology*, **1**, 784-791.

21. Meisenzahl, A.C., Shapiro, L. and Jenal, U. (1997) Isolation and Characterization of a Xylose-Dependent Promoter from *Caulobacter crescentus*. *Journal of Bacteriology*, **179**, 592-600.

22. Ouimet, M.-C. and Marczynski, G.T. (2000) Analysis of a cell-cycle promoter bound by a response regulator. *Journal of Molecular Biology*, **302**, 761-775.

23. Harlocker, S.L., Bergstrom, L. and Inouye, M. (1995) Tandem Binding of Six OmpR Proteins to the ompF Upstream Regulatory Sequence of Escherichia coli. *J. Biol. Chem.*, **270**, 26849-26856.

24. Forst, S.A., Delgado, J. and Inouye, M. (1989) DNA-binding properties of the transcription activator (OmpR) for the upstream sequences of ompF in *Escherichia coli* are altered by *envZ* mutations and medium osmolarity. *Journal of Bacteriology*, **171**, 2949-2955.

25. Rampersaud, A., Harlocker, S.L. and Inouye, M. (1994) The OmpR Protein of *Escherichia coli* Binds to Sites in the *ompF* Promoter Region in a Hierarchical Manner Determined by its Degree of Phosphorylation. *The Journal of Biological Chemistry*, **269**, 12559-12566.

26. Judd, E.M., Ryan, K.R., Moerner, W.E., Shapiro, L. and McAdams, H.H. (2003) Fluorescence bleaching reveals asymmetric compartment formation prior to cell division in *Caulobacter*. *Proc Natl Acad Sci U S A*, **100**, 8235-8240.

27. Kelly, A.J., Sackett, M.J., Din, N., Quardokus, E. and Brun, Y.V. (1998) Cell cycle-dependent transcriptional and proteolytic regulation of FtsZ in *Caulobacter*. *Genes and Development*, **15**, 880-893.

28. Freeman, J.M., Plasterer, T.N., Smith, T.F. and Mohr, S.C. (1998) Patterns of genome organization in bacteria. *Science*, **279**, 1827a.

29. Jones, S.E., Ferguson, N.L. and Alley, M.R.K. (2001) New member of the *ctrA* regulon: the major chemotaxis operon in *Caulobacter* is CtrA dependent. *Microbiology*, **147**, 949-958.

30. Reisenauer, A., Jensen, R.B. and Shapiro, L. (2002) DNA methylation affects the cell cycle transcription of the CtrA global regulator in *Caulobacter*. *The EMBO Journal*, **21**, 4969-4977.

31. Holtzendorff, J., Hung, D., Brende, P., Reisenauer, A., Viollier, P.H., McAdams, H.H. and Shapiro, L. (2004) Oscillating Global Regulators Control the Genetic Circuit Driving a Bacterial Cell Cycle. *Science*, **304**, 983-987.

32. Kumar, A., Grimes, B., Fujita, N., Makino, K., Mallock, R.A., Hayward, R.S. and Ishihama, A. (1994) Role of the Sigma^{70} Subunit of *Escherichia coli* RNA Polymerase in Transcription Activation. *Journal of Molecular Biology*, **235**, 405-413.

33. Jacobs, C., Ausmees, N., Cordwell, S.J., Shapiro, L. and Laub, M.T. (2003) Functions of the CckA histidine kinase in *Caulobacter* cell cycle control. *Molecular Microbiology*, **47**, 1279-1290.

34. Hung, D.Y. and Shapiro, L. (2002) A signal transduction protein cues proteolytic events critical to Caulobacter cell cycle progression. *Proc Natl Acad Sci U S A*, **99**, 13160-13165.

35. Stephens, C.M., Zweiger, G. and Shapiro, L. (1995) Coordinate cell cycle control of a *Caulobacter* DNA methyltransferase and the flagellar genetic hierarchy. *Journal of Bacteriology*, **177**, 1662-1669.

36. Stephens, C.M., Reisenauer, A., Wright, R. and Shapiro, L. (1996) A cell cycle-regulated bacterial DNA methyltransferase is essential for viability. *Proc. Natl. Acad. Sci. USA*, **93**, 1210-1214.

37. Mettke, I., Fiedler, U. and Weiss, V. (1995) Mechanism of activation of a response regulator: interaction of NtrC-P dimers induces ATPase activity. *J Bacteriol*, **177**, 5056-5061.

TRANSITION II

The analysis of CtrA/DNA interactions in Chapter II revealed that the form of CtrA consensus binding site clearly dictates a particular mode of CtrA binding. A third, low affinity, mode of CtrA binding at the *ctrA* promoters are still cell cycle regulated when a non-proteolyzable mutant of CtrA (CtrAΔ3) continuously occupies these promoters. This suggests new biochemical properties are needed to account for CtrA activity at the *ctrA* promoter. We also extend this observation to work published by Dr. Rania Siam who showed that a constitutively active CtrA mutant (CtrA D51E) demonstrated an unexpected low affinity for CtrA binding sites at *Cori* and yet provided all necessary functions *in vivo*. Therefore, constitutive CtrA occupation of a promoter (like the *ctrA* promoter) or a change in the CtrA protein itself (D51E) that disrupts DNA affinity, failed to demonstrate the expected change in phenotype suggesting these other properties may have been preserved.

Chapter III begins to evaluate how the temporal occupation of CtrA at the *Caulobacter* origin of replication is regulated and whether CtrA occupation at *Cori* influences the recruitment of other factors important for the coordination of replication initiation. In doing so I hope to begin to uncover those "other properties" alluded to Chapter II and to develop a better understanding of how a response regulator executes its function *in vivo*.

Chapter III: Recruitment of ClpX to a Chromosome Replication Origin

Authors: William J. Spencer & Gregory T. Marczynski*

Address: Department of Microbiology and Immunology
McGill University
Lyman Duff Building
3775 University Street
Montreal, Quebec, CANADA
H3A 2B4

*-corresponding author

ABSTRACT

In dividing cells, chromosome replication is restricted to once per cell division suggesting repression and activation of DNA replication is achieved through alterations in the three-dimensional state of protein/DNA complexes. Therefore, coordination of nucleoprotein dynamics with DNA replication suggests a higher level of regulation and the identification of chromosome remodeling factors such as protein chaperones are of great biological significance. The dimorphic bacterium *Caulobacter crescentus* displays a developmentally-regulated cell cycle and mode of replication reminiscent of eukaryotes and their more sophisticated developmental program (1). Replication in *Caulobacter* is regulated by the Clp/Hsp100 chaperone ClpX that directs the turnover of the origin binding protein CtrA (Figure 2A) (2), a repressor of chromosome replication (3). We provide *in vivo* evidence that ClpX transiently engages the chromosome replication origin (*Cori*) at the G1 to S transition and that ClpX-*Cori* interactions are CtrA-dependant since increased CtrA binding enhances ClpX recruitment. Further, this complex precedes the release of CtrA from *Cori*, providing a brief window during which replication complexes can assemble at *Cori* and initiate chromosome replication. These data, in combination with recent work (4), provide strong evidence that the preassembly of protein complexes, hence the rearrangement of chromatin structure by chaperones is a necessary step in the commitment to DNA replication.

INTRODUCTION

Chromosome replication is primarily controlled during initiation. Although the control mechanisms for chromosome replication are not clearly understood, current models predict that replication alternates between states that first promote and then repress chromosome replication in coordination with cell growth and development. Therefore, replication initiation requires "switch-factors" to supply essential activities for remodeling proteins at replication origins. Chaperones have been reported to play an important role in plasmid and phage replication. For example, the ClpX chaperone promotes RK2 plasmid replication by disaggregating dimers of the replication protein TrfA to the active monomer state (5). Replication initiation in λ requires a phage-encoded O-protein that recruits the λ P protein in complex with the DnaB helicase, to ori-λ (6). However, DnaB activity is suppressed by the λ P protein. The DnaK chaperone functions to releases P thereby stimulating helicase activity and subsequent DNA replication (7). In Mu phage, DNA replication is preceded by a unique transposition event requiring the MuA transposase (8). The ClpX chaperone helps stimulate Mu transposition by targeting MuA repressor for proteolysis by ClpP (9). Upon transposition, ClpX remodels the MuA-DNA complex promoting a shift from transposition to replication initiation (10,11). Therefore ClpX displays separate activities *in vivo*; One to promote proteolysis by targeting substrates to the ClpP protease (λ-O) and the second as an independent chaperone altering the activity of replication proteins (ie MuA, TrfA). However a role for chaperones in controlling chromosome replication remains incomplete.

The aquatic bacterium *Caulobacter crescentus* is an important cell cycle model to address how the coordination of DNA replication is coupled with cell cycle development (Figure 1). Chromosome replication in *Caulobacter* is regulated through positive and negative mechanisms originating at its single origin of replication (*Cori*) (Figure 1B). DNA replication (S-phase) is restricted to stalked cells (Fig. 1A) and depends on the essential activity of the initiator protein DnaA (12). Consequently, replication in *Caulobacter* occurs once per cell cycle (13) and repression is achieved in swarmer cells by the response regulator CtrA. Binding sites for both CtrA and DnaA are present within *Cori* (Fig. 1B) and the opposing activities of CtrA and DnaA are coordinated by periodic proteolysis through ClpP (2,14). Cell division produces two morphologically distinct progeny, the swarmer and stalked cell types whose underlying genetic programs are also distinct. Swarmer cells inherit a chromosome which cannot initiate replication while the stalk cell chromosome quickly re-initiates replication. This basic difference in replication potential between swarmer and stalk cells suggests the capacity to initiate replication is based on the inheritance of a specific nucleoprotein complex. As mentioned above, repression of chromosome replication at *Cori* is coordinated in swarmer cells by the response regulator CtrA (Fig. 1A) (15) and consequently binding to *Cori* by CtrA is not uniform (16) suggesting CtrA-*Cori* complexes may form a unique chromosome domain *in vivo* (inhibisome). Disassembling of this CtrA-Cori complex at the start of S-phase may represent an important cue for the initiation of chromosome replication.

Surprisingly, the over-expression of a non-proteolyzable variant of CtrA, CtrAΔ3, does not perturb the cell cycle or disrupt initiation of chromosome replication (3). These results imply that CtrA proteolysis is a dispensable feature for controlling chromosome replication. In this study we employ a chromatin-immunoprecipitation assay using formaldehyde crosslinking to investigate the temporal binding of CtrA and CtrAΔ3 to *Cori in vivo*. We have found that CtrA is dynamically localized to Cori and CtrA dissociation from Cori coincides with the swarmer to stalk transition step and the commencement of S-phase. Of significant interest is the recruitment of the molecular chaperone ClpX to Cori prior to S-phase.

MATERIALS AND METHODS

Chromatin Immunoprecipitation

Caulobacter cells were grown at 30°C in minimal media and synchronized by differential centrifugation in a ludox silica matrix (Sigma-Aldrich) (). Cells were released into fresh media at a density of OD660 = 0.2 and allowed to progress through the cell cycle. ChIP assays were performed based on previously published reports. In brief, approximately 2 OD_{660} units of cells were incubated with 1% formaldehyde buffered in 100mM sodium phosphate (pH 7.6). Cells were incubated at room temperature for 10 min and placed on ice for 30 min. Cells were collected by centrifugation and washed twice with sodium phosphate buffer (0.1M, pH 7.6) to remove excess formaldehyde. Cells were resuspended in BugBuster cell lysis reagent (Novagen) and incubated at 37°C for 30 minutes. Cells were washed with 2X IP wash buffer (100 mM Tris pH 7, 300 mM NaCl, 2% Triton X-100) along with PMSF (1mM final concentration) and incubated an additional 10 minutes at 37°C. Cell lysis was confirmed by microscopy. Lysates were subsequently sonicated three times (ten second pulses at 40% power output) on ice to reduce genomic DNA to 500-1000bp fragments. Samples were centrifuged and split into immunoprecipitation (IP) and Mock IP fractions. CtrA or ClpX rabbit IgG was added to each IP sample and incubated overnight at 4°C. 25ul of a 50% slurry of protein A sepharose (Sigma-Aldrich) was added to both the IP and Mock IP fractions. Samples were incubated 1 hour at room temperature with gentle rocking. Sepharose beads were washed five times with 1X IP wash buffer and twice with TE Buffer pH 8.0 (10 mM Tris, 1 mM EDTA, pH 8.0). Washed beads were resuspended in 50ul TE buffer and formaldehyde cross links were

reversed by heating for six hours at 65°C. Samples were collected by centrifugation and PCR was performed without further processing.

Western Blotting

CtrA and ClpX western blots were performed using standard denaturing 10% SDS-PAGE and transfer to PVDF (HyBond, GE Healthcare). Membranes were blocked in 5% non-fat skim milk in tris buffered saline (TBS) and immunoblots were carried out using a 1:5000 dilution of the CtrA rabbit polyclonal primary antibody, provided by Lucy Shapiro, Stanford California, a 1:10,000 dilution of ClpX primary antibody provided by Urs Jenal, Biozentrum, Basil Switzerland. Primary was conjugated with a 1:10,000 dilution of the goat anti rabbit HRP secondary (Chemicon). Membranes were washed repeatedly in fresh TBS buffer and developed using ECL+ immunoblotting detection reagents (GE Healthcare).

Primers and PCR

The oligonucleotides used to amplify *Cori* are as follows; *Cori* ChIP Primer A (5'-TTGAAGGAGGGAGCGGAAGG-3'); *Cori* ChIP Primer B (5'-TTGTCCAAAGACGCGGAACG-3') which amplifies a 223bp region in *Cori*. We quantitated the relative amount of input DNA from IP and Mock IP fractions using a reference DNA carrying a 48bp deletion from a plasmid cloned *Cori* fragment (Fig. 2A). We spiked all PCR reactions with serial dilutions of the competitor to determine the ration of the IP signal to Mock IP signal. Densitometry scans of the PCR gels were

performed and the results plotted as relative signal to reference DNA concentration. The ratio of the slopes for the IP and Mock IP signals were plotted as histograms over the period of the cell cycle measured (see Figure 3).

RESULTS AND DISCUSSION

In *Caulobacter*, CtrA and *Cori* are co-localized to the cell poles during the swarmer to stalk transition (17-19) at which time CtrA is cleared from *Cori* prior to DNA replication. Two models account for the loss of CtrA localization at Cori. One, proteolysis of unbound CtrA shifts the equilibrium of *Cori*-bound CtrA towards the unbound state. Two, CtrA is displaced from *Cori* by a remodeling factor that is able to enter *Cori* and destabilize CtrA prior to replication initiation (Figure 2B). Since ClpX is the chaperone responsible for directing CtrA proteolysis we wished to understand whether ClpX and CtrA colocalize to *Cori in vivo*. Chromatin immunoprecipitation (ChIP) assays allow for sensitive detection of protein-DNA complexes by cross-linking protein/DNA complexes in growing cells. During ChIP assays, complexes are recovered by immuno-antibody precipitation and the specific DNA sequences are identified by polymerase chain reaction (PCR). Using ChIP assays we measured the *in vivo* occupancy of both CtrA and ClpX at *Cori* in synchronized cell populations. Our data show that CtrA occupies Cori *in vivo* (Fig. 4A) and tracks the bulk protein concentration in the cells (Fig. 4B). At the commencement of the cell cycle (t=0, Figure 4A), we observe strong binding of CtrA to *Cori* in swarmer cells followed by a rapid loss of CtrA binding to *Cori* at the swarmer to stalk transition phase, coincident with the start of DNA replication begins (t= 30, 45 min; Fig 4A). This change in CtrA binding to *Cori* parallels protein abundance as measured by western blot analysis (Figure 4B). *Cori* remains free of CtrA for an approximate period of 45 minutes. This window while sufficient for the initiation of replication suggests control of re-replication during this period may be under a CtrA-independent mechanism such as DnaA inactivation by RIDA (Chapter 4). The resynthesis of CtrA

protein in predivisional cells (t= 60) coincides with the reassociation of CtrA to *Cori.* However ClpX protein, which is present throughout the cell cycle (Fig. 4B) and evenly distributed in both the replicating and non-replicating cell types only contacts *Cori* in swarmer cells prior to the start of S-phase (t= 0, 15 min: Figure 4A). The results in Figure 3 suggest ClpX binding to *Cori* may be CtrA dependent. To address the role of CtrA in recruiting ClpX to *Cori* we expressed the non-proteolyzable CtrAΔ3 allele in wild-type cells. This protein binds *Cori* with higher affinity (Fig. 5A) and is expressed throughout the cell cycle (Fig. 5B). In swarmer cells, CtrAΔ3 is expressed in combination with wild type CtrA and shows a considerable increase in *Cori* binding over WT cells alone (Figure 4A). However, regardless of the persistence of CtrAΔ3, the protein is 'actively' displaced from *Cori* at the commencement of chromosome replication (t= 55 min: Figure 5A). Although CtrAΔ3 is displaced, the CtrA-free window is reduced to less than 15 minutes. While cells grow normally under these conditions (data not shown), the increase in CtrAΔ3 binding to *Cori* in swarmer cells stimulates a corresponding increase in ClpX binding to *Cori,* providing strong evidence that ClpX recruitment to *Cori* in swarmer cells is likely CtrA-dependant.

Our data clearly show that CtrA and the ClpX chaperone interact with the *Caulobacter* origin of replication in a cell cycle dependent manner and that ClpX represents a new member of origin binding proteins. The data suggest that important remodeling events drive the transition from repression to activation of replication initiation and suggest CtrA is actively removed from *Cori* during the swarmer to stalk transition and this activity is not dependent CtrA proteolysis (i.e. CtrAΔ3). In this context, it is ClpX chaperone

activity and not proteolysis that provides the necessary activity required to clear *Cori* of CtrA and permit replication initiation. It is not clear why so many CtrA binding sites are found within *Cori* (5 binding sites) but CtrA binding to *Cori* is not uniform (16) and suggests the three dimensional characteristics of a CtrA-*Cori* complex may be ordered asymmetrically. A similar asymmetry model has also been proposed for the MuA transposase (11) which forms asymmetric complexes with target DNA sequences. The MuA/DNA complexes are disassembled by ClpX to permit replication of Mu phage DNA. (20). Therefore chromosome replication would require the disassembly of the CtrA-*Cori* complex and we suggest ClpX could serve this role. Recent work identifies that ClpX and CtrA colocalize at the stalk pole through a newly identified specificity factor RcdA (4) and previous work has also shown that *Cori* is component of the stalk pole complex (19). The work presented here provides an important link between targeted protein complex assembly and *Cori* replication. These data also suggests that the chromosome origin is remodeled throughout the cell cycle and it is through these remodeling events that chromosome replication is permitted.

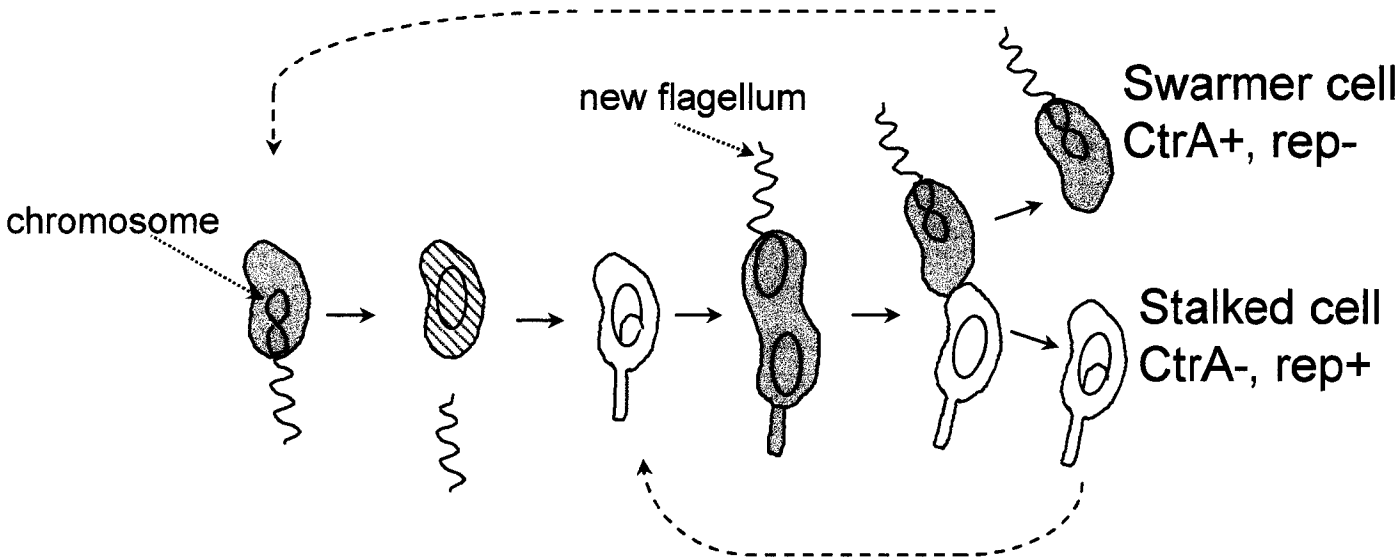
We further suggest that CtrA-mediated recruitment of ClpX to *Cori* is not only required for altering CtrA occupancy at *Cori* but may stimulate contact with other origin binding proteins such as the initiator protein DnaA. *Caulobacter crescentus* displays a well defined cell cycle which couples DNA physiology with cellular morphology and restricts chromosome replication to once per cell cycle (13). The conspicuous appearance of the ClpX chaperone at a unique stage in the cell cycle (G1-S) at a specific region on the chromosome (*Cori*) reveals a novel feature of replication initiation in *Caulobacter*. The

conserved nature of ClpX and chaperones in general among eubacteria suggests similar remodeling activities are present. Consequently, in *Caulobacter*, *Cori* independently supports autonomous plasmid replication (21). Paradoxically these plasmids over replicate suggesting cell cycle controls are relaxed because specific features, present in the chromosome, are missing from these plasmids or as I suggest, a specific nucleoprotein structure is not faithfully inherited. Future work on the chromatin dynamics of the *Caulobacter* chromosome may help address this paradox.

FIGURES

Figure 1. A) The *Caulobacter crescentus* cell cycle. The cycle begins with a non-replicating chemotactic swarmer cell (Sw) which differentiates to the replicating stalk cell (St). Growth of the pre-divisional cell produces a new flagellated swarmer pole. Segregating chromosomes are positioned in both the non-replicating swarmer (rep-) and the replication competent stalk cell (rep+). Shading indicates the temporal and spatial presence of the CtrA response regulator. ChIP primers indicated the relative position of the primer pair used to track *in vivo* occupation of CtrA at *Cori*. B) Conspicuous features of the *Caulobacter* Origin of Replication (Cori). The five iterative CtrA binding sites, a single IHF binding site near CtrA binding site c as well as a putative DnaA box in the rightward half of Cori (open arrow) are shown. Cori is flanked by two open reading frames, *hemE* and *Duf299* along with three promoters, weak (Pw), strong (Ps) and P3. Also shown is the AT rich sequence where melting of the origin DNA occurs.

A


new flagellum
Swarmer cell
CtrA+, rep-
chromosome
Stalked cell
CtrA-, rep+

B

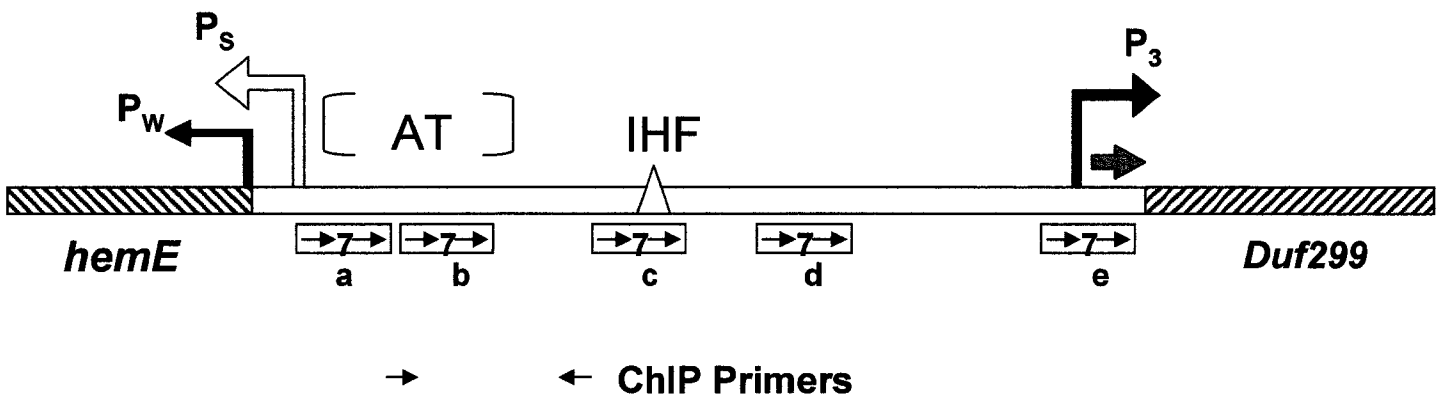

P_S
P_W
AT
IHF
P_3
hemE
a
b
c
d
e
Duf299
ChIP Primers

Figure 2. *ClpX and the regulation of CtrA proteolysis.* A) In stalk cells, the hexameric chaperone ClpX targets CtrA through a recognition domain found in the C-terminal domain of CtrA, unwinds CtrA through the core of the ClpX ring structure and feeds the linearized protein into the ClpP protease. The CtrAΔ3 allele has a specific disruption in the ClpX recognition motif which prevents Clp-dependent degradation in stalk cells. B) Proposed model of ClpX-mediated dissociation of CtrA from *Cori*. The first model (top) suggests ClpX can directly contact CtrA when it is bound to sites within *Cori* and remove it for degradation. The second model (bottom) suggests *Cori*-bound CtrA is not accessible (closed) and that the degradation of unbound CtrA drives the dissociation of CtrA from *Cori*.

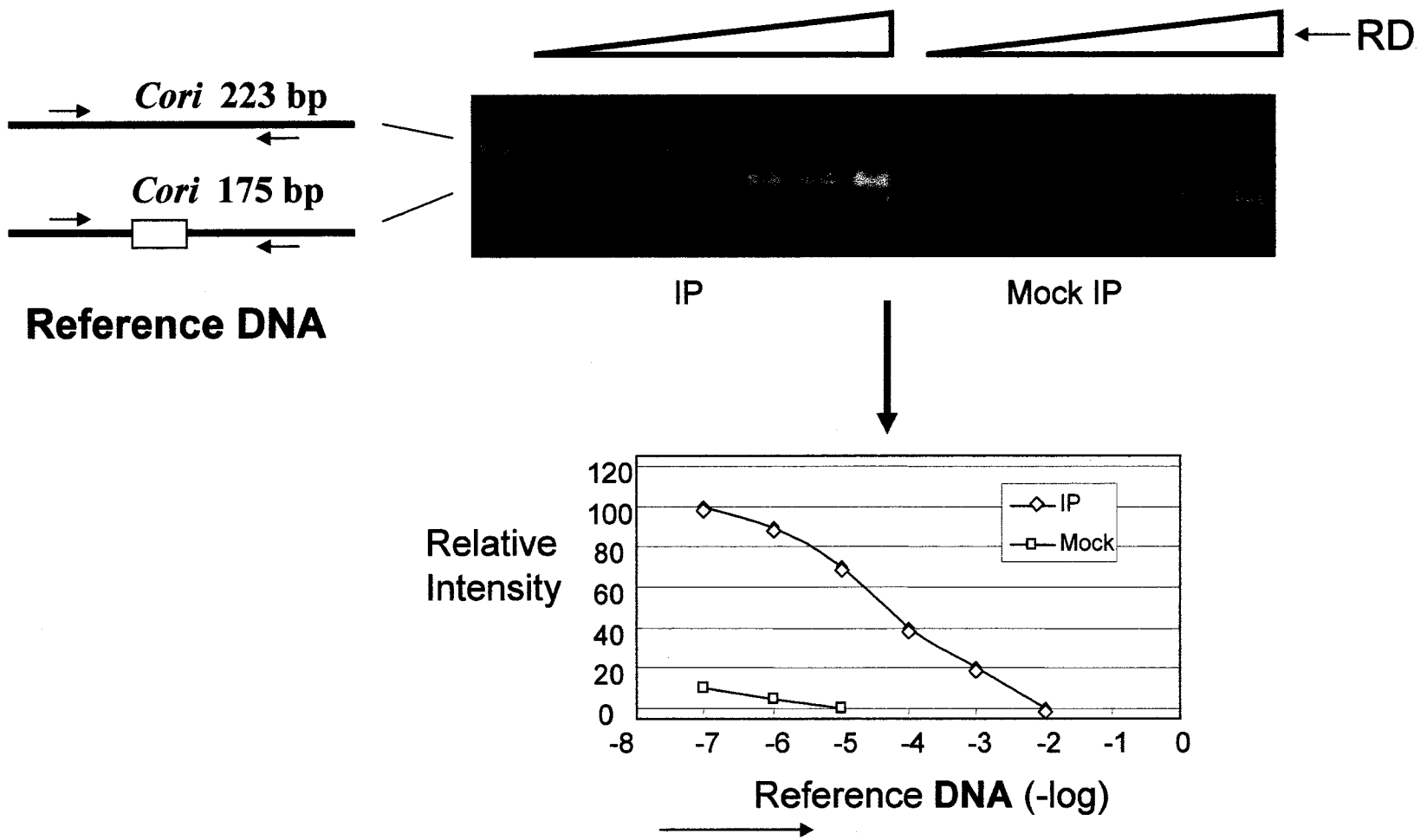
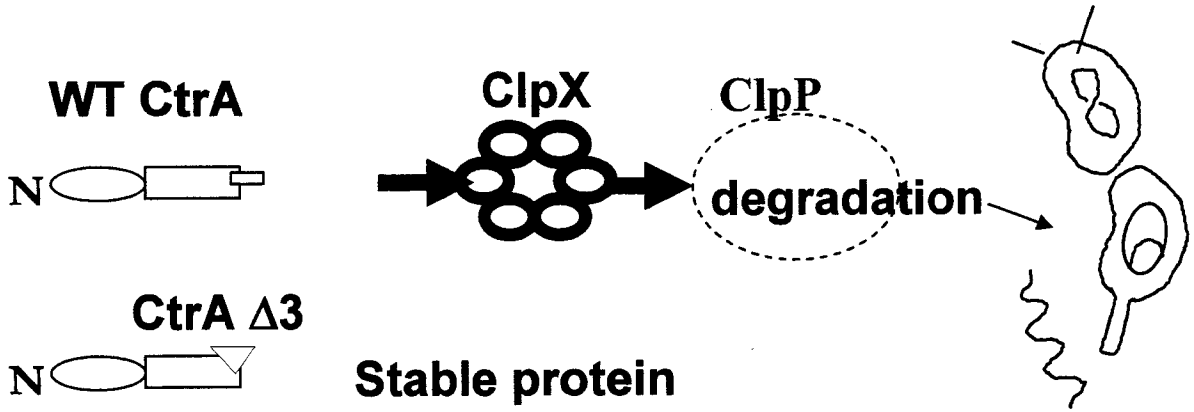

RD
Cori 223 bp
Cori 175 bp
Reference DNA
IP
Mock IP
Relative Intensity
120
100
80
60
40
20
0
-8
-7
-6
-5
-4
-3
-2
-1
0
IP
Mock
Reference DNA (-log)

Figure 3. Quantitation procedure for determining enrichment of the *Cori* signal in immunoprecipitated (IP) and mock immunoprecipitated (mock IP) fractions of synchronized *Caulobacter* cell lysates. Mock IP and IP fractions were amplified by polymerase chain reaction. Both IP and Mock IP samples were spiked with an increasing dose of a reference DNA (RD) carrying a 48 bp deletion in *Cori* (top left panel). Both the target and reference DNA are recognized by the same primer set and therefore the reactions are competitive. Samples were separated by agarose gel electrophoresis (top right panel) and digitally images were quantitated. The relative pixel density of each band was determined and plotted as function of reference DNA concentration (bottom panel). For the histograms reported in Figure 3 and Figure 4 the relative PCR signal is the ratio of the slope for the IP and Mock IP competition curves.

A


WT CtrA
N
ClpX
ClpP
degradation
CtrA Δ3
N
Stable protein

B

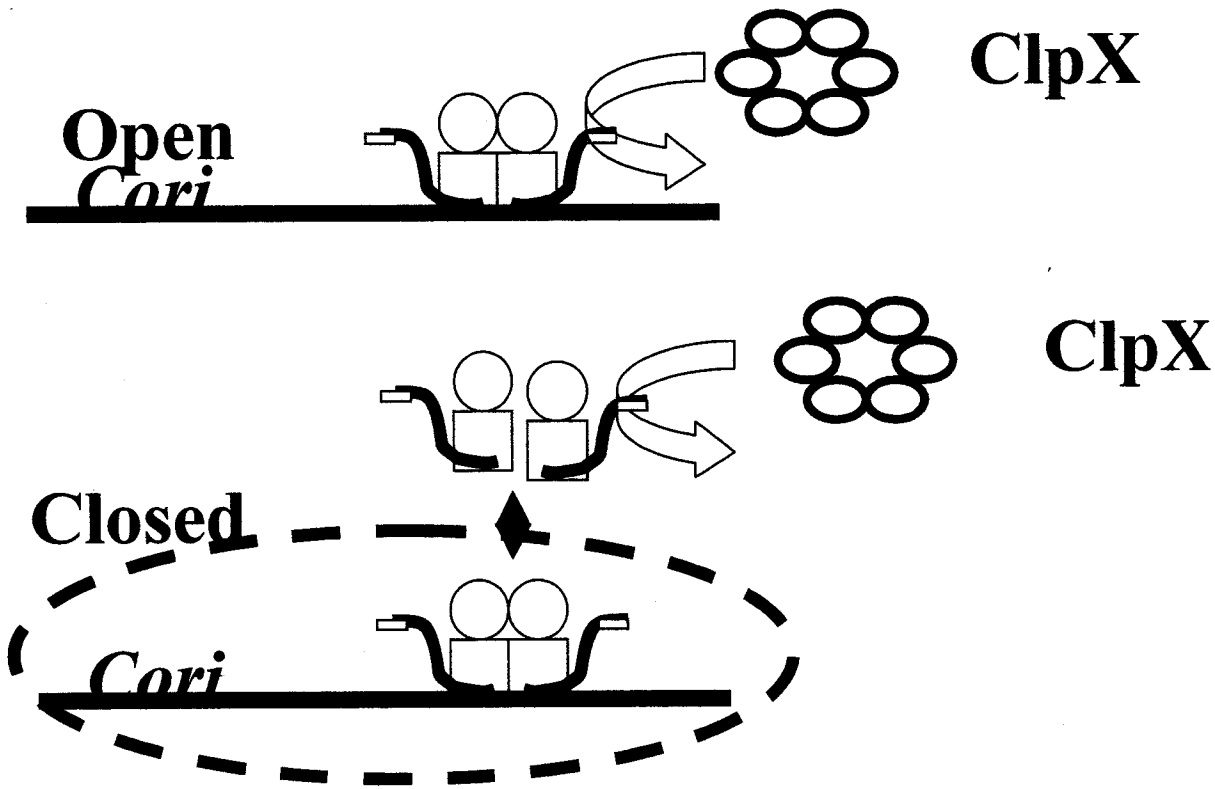
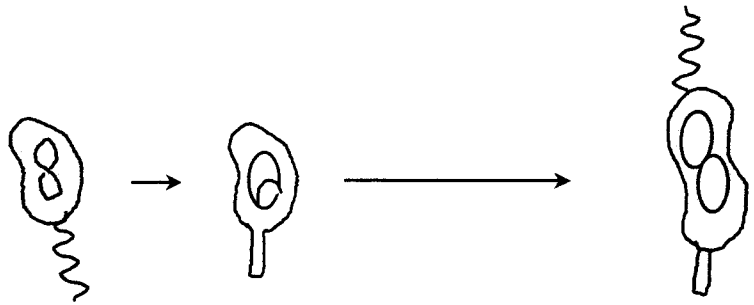
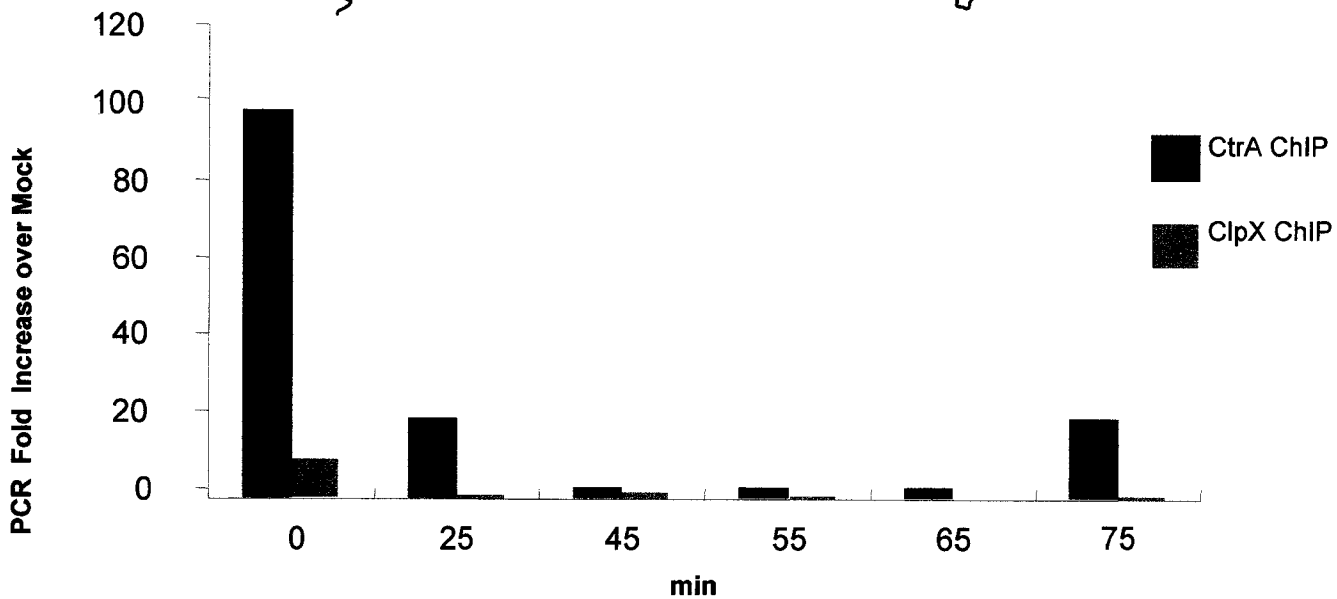

ClpX
Open
Cori
ClpX
Closed
Cori

Figure 4. *CtrA and ClpX protein crosslinking to the chromosome replication origin (Cori) DNA during the Caulobacter cell cycle.* (A) Chromatin immunoprecipitation (ChIP) assays of CtrA and ClpX in WT *Caulobacter* cells. Histogram shows the fold increase of the immunoprecipitated PCR signal over mock immunoprecipitated PCR signals for each time point (min) during the cell cycle. (B) Western blots indicate the cell cycle abundance of CtrA and ClpX.

A




120
100
80
60
40
20
0
PCR Fold Increase over Mock
0
25
45
55
65
75
min
CtrA ChIP
ClpX ChIP

B

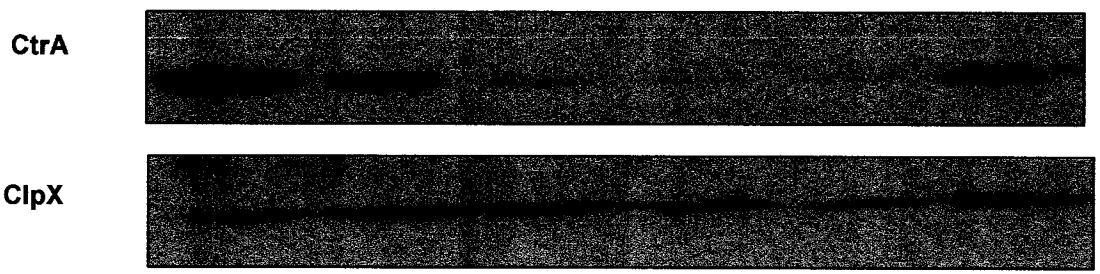
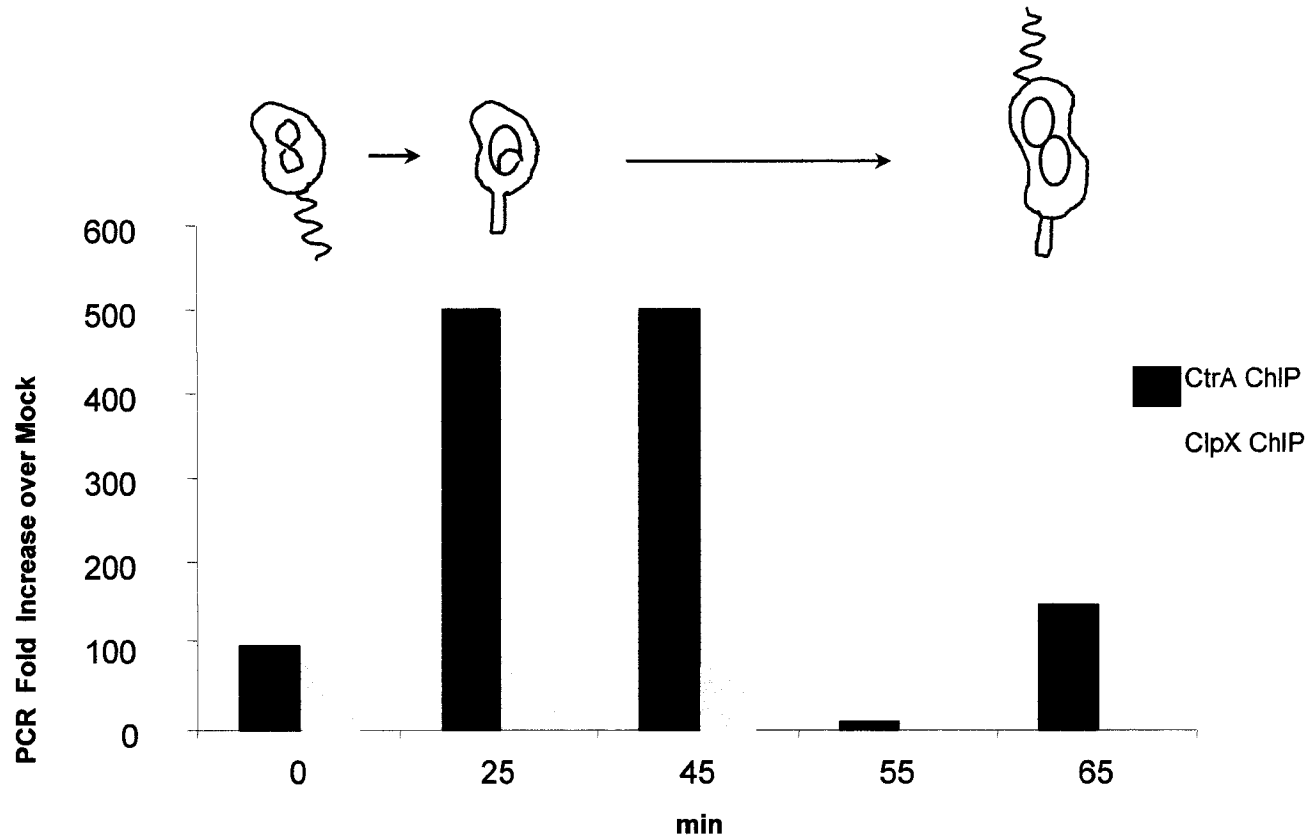
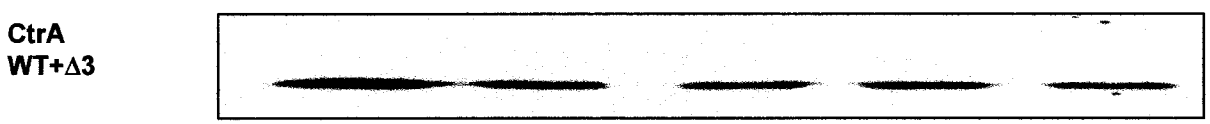

CtrA
ClpX

Figure 5. CtrAΔ3 and ClpX protein crosslinking to replication origin (Cori) during the *Caulobacter* cell cycle. (A) Chromatin immunoprecipitation (ChIP) assays of CtrA and ClpX in WT *Caulobacter* cells expressing CtrAΔ3 non-proteolyzable allele. Histogram shows the fold increase of the immunoprecipitated PCR signal over mock immunoprecipitated PCR signal for each time point (min) during the cell cycle. (B) Western blots showing the combined cell cycle abundance of WT CtrA and CtrAΔ3.

A


600
500
400
300
200
100
0
PCR Fold Increase over Mock
0
25
45
55
65
min
CtrA ChIP
ClpX ChIP

B


CtrA
WT+Δ3

REFERENCES

1. Marczynski, G.T. and Shapiro, L. (2002) Control of chromosome replication in *Caulobacter crescentus*. *Annual Reviews in Microbiology*, **56**, 625-656.

2. Jenal, U. and Fuchs, T. (1998) An essential protease involved in bacterial cell cycle control. *The EMBO Journal*, **17**, 5658-5669.

3. Domian, I.J., Quon, K.C. and Shapiro, L. (1997) Cell type-specific phosphorylation and proteolysis of a transcriptional regulator controls the G1 to S transition in a bacterial cell cycle. *Cell*, **90**, 415-424.

4. McGrath, P.T., Iniesta, A.A., Ryan, K.R., Shapiro, L. and McAdams, H.H. (2006) A Dynamically Localized Protease Complex and a Polar Specificity Factor Control a Cell Cycle Master Regulator. *Cell*, **124**, 535-547.

5. Konieczny, I. and Helinski, D.R. (1997) The replication initiation protein of the broad-host-range plasmid

RK2 is activated by the ClpX chaperone. *PNAS*, **94**, 14378-14382.

6. Dodson, M., Roberts, J., McMacken, R. and Echols, H. (1985) Specialized nucleoprotein structures at the origin of replication of bacteriophage lambda: complexes with lambda O protein and with lambda O, lambda P, and Escherichia coli DnaB proteins. *Proc Natl Acad Sci U S A*, **82**, 4678-4682.

7. Dodson, M., Echols, H., Wickner, S., Alfano, C., Mensa-Wilmot, K., Gomes, B., LeBowitz, J., Roberts, J.D. and McMacken, R. (1986) Specialized nucleoprotein structures at the origin of replication of bacteriophage lambda: localized

unwinding of duplex DNA by a six-protein reaction. *Proc Natl Acad Sci U S A*, **83**, 7638-7642.

8. Baker, T.A., Mizuuchi, M. and Mizuuchi, K. (1991) MuB Protein Allosterically Activates Strand Transfer by the Transposase of Phage Mu. *Cell*, **65**, 1003-1013.

9. Jones, J.M., Welty, D.J. and Nakai, H. (1998) Versatile action of *Escherichia coli* ClpXP as protease or molecular chaperone for bacteriophage Mu transposition. *J Biol Chem*, **273**, 459-465.

10. Kruklitis, R., Welty, D.J. and Nakai, H. (1996) ClpX protein of Escherichia coli activates bacteriophage Mu transposase in the strand transfer complex for initiation of Mu DNA synthesis. *EMBO Journal*, **15**, 935-944.

11. Burton, B.M. and Baker, T.A. (2006) Remodeling protein complexes: Insights from the AAA+ unfoldase ClpX and Mu transposase. *Protein Science*, **14**, 1945-1954.

12. Gorbatyuk, B. and Marczynski, G.T. (2001) Physiological consequences of blocked *Caulobacter crescentus* DnaA expression, an essential DNA replication gene. *Molecular Microbiology*, **40**, 485-497.

13. Marczynski, G.T. (1999) Chromosome methylation and the measurement of faithful, once and only once per cell cycle chromosome replication in *Caulobacter crescentus*. *Journal of Bacteriology*, **181**, 1984-1993.

14. Gorbatyuk, B. and Marczynski, G.T. (2005) Regulated degradation of chromosome replication proteins DnaA and CtrA in Caulobacter crescentus. *Mol Microbiol*, **55**, 1233-1245.

15. Quon, K.C., Yang, B., Domian, I.J., Shapiro, L. and Marczynski, G.T. (1998) Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin. *Proc. Natl. Acad. Sci. USA*, **95**, 120-125.

16. Siam, R. and Marczynski, G.T. (2000) Cell cycle regulator phosphorylation stimulates two distinct modes of binding at a chromosome replication origin. *The EMBO Journal*, **19**, 1138-1147.

17. Iniesta, A.A., McGrath, P.T., Reisenauer, A., McAdams, H.H. and Shapiro, L. (2006) A phospho-signaling pathway controls the localization and activity of a protease complex critical for bacterial cell cycle progression. *Proc Natl Acad Sci U S A*, **103**, 10935-10940.

18. Ryan, K.R., Huntwork, S. and Shapiro, L. (2004) Recruitment of a cytoplasmic response regulator to the cell pole is linked to its cell cycle- regulated proteolysis. *Proc Natl Acad Sci U S A*, **101**, 7415-7420.

19. Jensen, R.B., Wang, S.C. and Shapiro, L. (2001) A moving DNA replication factory in *Caulobacter crescentus*. *The EMBO Journal*, **20**, 4952-4963.

20. Burton, B.M. and Baker, T.A. (2003) Mu Transpososome Architecture Ensures that Unfolding by ClpX or Proteolysis by ClpXP Remodels but Does Not Destroy the Complex. *Chem Biol*, **10**, 463-472.

21. Marczynski, G.T. and Shapiro, L. (1992) Cell-cycle Control of a Cloned Chromosomal Origin of replication from *Caulobacter crescentus*. *Journal of Molecular Biology*, **226**, 959-977.

TRANSITION III

The data presented in Chapters III revealed that *Caulobacter* utilizes the chaperone, ClpX, to possibly remodel CtrA at the replication origin and prepare the chromosome for replication. In the absence of CtrA, the control of over-replication is of paramount importance because newly replicated origins and the factors that promote replication (i.e. DnaA) must be compartmentalized. In Chapter III, we saw that CtrA does not occupy *Cori* in early stalk cells when chromosome replication initiates. It has been well established that chromosome replication is negatively regulated by CtrA in swarmer cells however it is not clear what role CtrA plays in preventing re-replication in stalk cells. Chapter III demonstrates that CtrA is absent from *Cori* during the replicative stalked cell stage. This strongly suggests that other mechanisms are required to prevent re-replication in stalk cells. A genomic search for factors that might control replication revealed a surprising lack of the regulatory systems utilized by *E. coli*. For instance, homologs of SeqA (sequestration model) or the *datA* locus (titration model) are unknown. In *E. coli,* DnaN (beta clamp) and Hda (Homolog of DnaA) are part of a regulatory mechanism called Regulated Inactivation of DnaA (RIDA) and prevents re-replication by turning off DnaA activity. In *Caulobacter* both homologs of DnaN and Hda are present including a novel DnaA-like gene that appears restricted to the alpha-proteobacteria group. Chapter IV demonstrates that the components of RIDA are present and suggests *Caulobacter* may utilize RIDA to control chromosome replication.

CHAPTER IV: CHARACTERIZATION OF TWO NOVEL DNAA-LIKE GENES

ABSTRACT

Escherichia coli prevents excess chromosome replication through three negative feedback mechanisms that reduce activity of the initiator protein DnaA and thereby limit chromosome replication to once per cell division. We show that the most important of these mechanisms, regulated inactivation of DnaA (RIDA) may be present in the aquatic bacterium *Caulobacter crescentus*. Isolation of two *Caulobacter* DnaA like genes (Cdl-1 and Cdl-2) genes show distinct homologies to the DnaA gene. Cdl-1, is homologous to the ATPase domain of the DnaA gene and the previously identified Hda, a component of RIDA in *E. coli*. The second gene, Cdl-2, is a small molecular weight protein (~14 kDa) restricted to the phylogenetic class of alpha-proteobacteria. Cdl-2 is homologous to the DNA binding domain of DnaA and suggests a novel mechanism (e.g. modified RIDA or another negative-feedback). Over-expression of either DnaA-homologue influences growth rate and cell morphology and suggests that precise levels of this protein are required for normal cell growth and development. These strains also show reduced levels of DNA biosynthesis suggesting Cdl-1 and -2 might function in a RIDA-like manner to limit DNA replication.

INTRODUCTION

In *E. coli*, replication appears to be controlled at the level of initiation and is repressed by three negative feedback mechanisms (Figure 1). The redundancy of these mechanisms ensures replication is properly synchronized and coordinated (1). Immediately following replication initiation from the *E. coli* origin (*oriC*), cells are unable to re-initiate due to rapid sequestration of the newly replicated origins (2). This process involves SeqA, a protein which confines the hemi-methylated origin regions of the chromosome to the membrane and after a prescribed length of time the origins are released, allowing cells to enter another round of replication (reviewed in (3). A second mechanism is a locus of high affinity DnaA boxes within the *E. coli* chromosome, called *datA,* which titrates the replication initiator protein DnaA (4). The final control mechanism of replication initiation in *E. coli* is the hydrolytic turnover of DnaA-ATP to DnaA-ADP which disrupts the ability of DnaA to function within OriC (5). DnaA-ATP hydrolysis appears to be mediated by a multi-component process called Regulated Inactivation of DnaA or RIDA that includes the highly conserved DnaA-like gene *hda* (see Table 2) (6,7) and the beta clamp of DNA polymerase III (8). The role of the beta-subunit in RIDA suggests the terminal stages of replisome assembly provide checkpoint inhibition of DnaA initiator function (9). The three mechanisms presented here, sequestration, titration, and RIDA, work in concert to control replication initiation in *E. coli*. Recent work suggests RIDA is the dominant mechanism controlling hyper-initiation (9). Using a quantitative DNA microarray analysis, Camara et al (2005) measured the ratio of *OriC*-to-terminus regions (a measure of over-replication) in E. coli strains carrying separate *seqA*, *datA*, and *hda*

mutations. They demonstrated that *hda*, rather than *seqA* or *datA* mutants show a significant increase in the ratio of *OriC*-to-terminus signal.

Caulobacter crescentus, which restricts the replication of its chromosome to once per cell, utilizes DnaA as a replication initiator (10). However, *Caulobacter* appears to lack the homologous features of SeqA and *datA*. The recent discovery of two DnaA-like genes, Cdl-1 and Cdl-2 (see Figure 1), suggests a RIDA-like mechanism may be utilized in *Caulobacter*. Each round of asymmetric cell division produces two distinct cell types, a non-replicating swarmer cell and a stalk cell that immediately re-initiates chromosome replication. While CtrA binding to the replication origin (*Cori*) accounts for repression of chromosome replication in swarmer cells, it is not clear how re-replication is prevented in stalk cells that have already initiated chromosome replication. Chapter 3 outlined that CtrA does not occupy *Cori in vivo* during early S-phase. This data suggests, a mechanism, other than CtrA binding to *Cori,* may function to limit chromosome replication in stalk cells. Here we show that *Caulobacter* requires precise levels of two DnaA-like genes Cdl-1 and Cdl-2 for proper cell cycle progression and over expression of either gene results in defects in cell division. The data demonstrate that DNA synthesis in these cells is significantly impaired, suggesting a RIDA mechanism exists in *Caulobacter*.

MATERIALS AND METHODS

Strains and Growth Conditions

All experimental strains were generated using the wild type NA1000, a synchronizable *Caulobacter* strain. All cultures were grown exponentially from OD_{660} = 0.1 at 30°C in minimal M2 media supplemented with either 0.2% glucose or 0.2% xylose. The open reading frames of the Cdl-1 (CC1711) and Cdl-2 (CC1058) were PCR amplified from genomic template DNA using Taq polymerase (GE Healthcare) and the following primer pairs (Invitrogen):

Table 1. PCR primers used to amplify Cdl-1 and Cdl-2

cc1711-5'	5'-ATCGGAATTCTTGTCCACCCAGTCCAAA-3'
cc1711-3'	5'-ATCGGAATTCCGTCACGGCCTACCCCTCATC-3'
cc1058-5'	5'-ATCGGAATTCATGCTGGTTCAAGCCCTT -3'
cc1058-3'	5'-ACTGGAATTCTTACGGCAGATCCTGAGC-3'

Subsequent PCR products were cloned into the pDRIVE-PCR vector (Qiagen) and transformants were screened by the standard blue/white selection which selects for disrupted LacZ activity by the cloned PCR fragment. Plasmid preps of positive transformants were screened by restriction digests and confirmed by DNA sequencing (Sheldon Biotechnology Centre). EcoRI fragments of both *cdl-1* and *cdl-2* were individually subcloned into the xylose inducible plasmid pUJ142 (11) and confirmed by restriction digestion. These plasmids were subsequently mobilized in *Caulobacter* NA1000 cells by conjugation with the *E. coli* strain S17-1 (12).

In silico Analysis

Sequence alignments of Cdl-1 and Cdl-2 homologs of alpha-proteobacteria species were originally identified through a BLAST search from TIGR using the Comprehensive Microbial Resource (CMR). Sequences were subsequently downloaded and re-aligned using the proprietary software Clone Manager™.

DNA Synthesis Assays

Growing *Caulobacter* cells were sampled and normalized to A660 = 0.1 and nucleotide incorporation rates measured. Mix 50 µl of *Caulobacter* cells in M2-glucose media with $2x10^6$ cpm of 32-P alpha dNTPs (GE Healthcare dCTP 5000 Ci/mmol). Incubate at RT for one minute and remove 20 µl and mix with 200 µl of 0.5 NaOH in a glass tube on ice. Heat tubes to 65oC for 30 min to remove RNA signal. Add 2 ml 20% trichloroacetic acid (TCA) and incubate on ice for 30 min. Collect precipitated material on glass fiber filters (Fisher Scientific) and wash tubes with excess 5% TCA. Wash filters with 95% ethanol, air dry and determined activity by scintillation counting.

Microscopy

During DNA synthesis reactions, growing cells were monitored by microscopy. 10 µl of cells were spotted onto glass slides (Fisher Scientific) and digitally photographed (magnification = 100X oil emersion) using a standard USB mounted digital camera and Zeiss phase contrast microscope. Captured images were edited using Adobe Photoshop software.

RESULTS

The Caulobacter Genome has two DnaA-like Genes

Recent work in *Escherichia coli* has identified Hda, a homolog of DnaA in *Escherichia coli*, as an apparent negative regulator of DnaA. Hda function is coordinated with the beta clamp of DNA polymerase III (8). Hda/beta-clamp inhibits the initiation of chromosome replication by stimulating the intrinsic nucleotide exchange activity of DnaA (7). We have also identified an Hda homologue (*cdl-1*) in the *Caulobacter* genome including a novel DnaA-like gene, *cdl-2*. Both Caulobacter *dnaA*-like genes have distinct homologies to the *dnaA* gene (Figure 1). The first, *cdl1*, is homologous to the ATPase domain of DnaA and we propose has similar functions to Hda *in E. coli*. The second gene identified, *cdl2*, is homologous to the DNA binding domain of DnaA and whose function is only speculative (see Discussion). Homologues of Cdl-1 are numerous and widely conserved (Table 2). Alignment of Hda homologues from other bacteria show that the Walker A motif (ATP binding domain) is highly conserved (Figure 2A). However, the Walker B motif (ATP hydrolysis domain) does not appear to be conserved among the alpha-proteobacteria (*Caulobacter* to *Maricaulis* inclusive). A second domain is also present and is an important feature of AAA+ ATPases. This domain includes the nucleotide interacting motifs Sensor I and II (13) that coordinate Walker B hydrolysis of ATP (14). The Box VII motif, which plays a role in Hda dimerization (15) and DnaA-ATP hydrolysis is also highly conserved.

Surprisingly, Cdl-2 homologues are restricted to the alpha-proteobacteria class (Figure 2B). The most conspicuous feature of this protein is the conservation of the DNA-binding domain that shares sequence homology with the DNA-binding domain of DnaA.

Cell Growth and Morphology

To address the role of Cdl-1 and Cdl-2, both genes were PCR amplified from genomic *Caulobacter* DNA and sub-cloned into the xylose-inducible pUJ142 vector (11). Wild-type *Caulobacter* cells (vector control) and a xylose-inducible CtrA mutant (CtrAD51EΔ3) that blocks chromosome replication (16) were used to compare the growth rates, morphology and DNA synthesis of Cdl-1 and Cdl-2 overexpressing cells. In Figure 3A, Cdl-1- and Cdl-2-expressing cells continue to grow exponentially however the cells become increasingly filamentous and non-motile (Figure 4: Xylose-3h). After 14 hours of growth in xylose, Cdl-2 cells show an outgrowth of cells with WT morphology (Figure 4). This observation suggested a loss of Cdl-2 expression because plasmids recovered from 14 hour Cdl-2 cells failed to induce a filamentous phenotype when reintroduced in WT *Caulobacter* (data not shown).

DNA Synthesis

The filamentous phenotype of Cdl-1 and Cdl-2-expressing cells implies a defect in cell division. Because DNA replication is a major checkpoint controlling cell division in *Caulobacter*, DNA synthesis of Cdl-1 and Cdl-2 overexpressing cells was investigated (Figure 3B). Upon induction with xylose, all cells show the anticipated lag in DNA synthesis because of the change in carbon source (glucose → xylose). DNA synthesis

continues to decline for CtrAD51EΔ3-expressing cells and is consistent with the lethality of this protein in cells. In glucose minimal media, prior to xylose induction, DNA synthesis in both Cdl-1 and Cdl-2 expressing cells is low and suggests precise levels of these proteins are required. Upon induction with xylose, a further reduction in DNA synthesis was observed.

DISCUSSION

Checkpoint control in *Caulobacter* drives cell cycle progression and cell division (17). A number of mechanisms are essential for regulating DNA replication in *Caulobacter*. Of primary focus is CtrA, that represses chromosome replication in swarmer cells (18). However in stalk cells, the degradation of CtrA protein and the loss of binding at *Cori* (Chapter 3) suggest CtrA is not available to suppress chromosome replication in stalk cells. The identification of RIDA homologues in *Caulobacter* suggests this mechanism functions to prevent re-replication in *Caulobacter*. In *E. coli,* regulatory redundancy ensures that DnaA activity is properly coordinated (19). The apparent absence of regulatory redundancy in *Caulobacter* (i.e. sequestration and titration) supports a role for RIDA.

The work presented here highlights the discovery of a new class of adaptor proteins in *Caulobacter* that have strong similarity with specific domains of the DnaA protein. Cdl-1 is homologous with the ATPase domain of *Caulobacter* DnaA including many of the regulatory sequences found among AAA+ super-family members. The proposed mechanism of RIDA in *E. coli* involves the formation of Hda homodimers through a conserved arginine residue (Box VII domain) (15). The conservation of Box VII in Hda's from other bacterial species (Figure 2A) suggests Cdl-1 dimerization may function in *Caulobacter*. Overexpression of Cdl-1 in cells suppresses DNA synthesis (Figure 3B) and causes distinct morphologies (Figure 4). These data support the proposed role of Cdl-1 as a repressor and the functional existence of RIDA in *Caulobacter*.

The homology of Cdl-2 to the DNA-binding domain of *Caulobacter* DnaA suggests that Cdl-2 may bind DNA (e.g. DnaA boxes). Our lab has begun to test whether Cdl-2 binds to a single DnaA box in the *Caulobacter* origin of replication (*Cori*). DNase I footprinting assays, using *Cori* and purified GST-Cdl-2, have not conclusively demonstrated that Cdl-2 binds DNA *in vitro* (data not shown). The restriction of cdl-2 homologues to alpha-proteobacteria species was unexpected and suggests Cdl-2 may function as part of a modified RIDA or a novel regulatory mechanism. Similar to Cdl-1, Cdl-2 overexpression alters cell morphology and suppresses DNA synthesis.

Future studies are needed to understand the essential nature of *cdl-1* and *cdl-2* and forthcoming gene knockouts will address this important question. If Cdl-1 or Cdl-2 directly regulate DnaA activity, experiments designed to measure the ratio of DnaA-ATP to DnaA-ADP can be developed (7). The *Caulobacter* Cell Cycle Microarray Project (see http://caulobacter.stanford.edu/CellCycle/) shows that Cdl-1 and Cdl-2 mRNAs appear to be down-regulated following the swarmer to stalk transition. These data suggest both proteins are available in stalk cells when chromosome replication commences. Further work on the cell cycle availability of these proteins should reveal how RIDA is regulated in *Caulobacter*. Perhaps the most novel feature of DnaA regulation in Caulobacter is its periodic proteolysis (20). How RIDA is coordinated with DnaA proteolysis is of considerable interest and we anticipate will provide new insights into how negative regulation of DnaA and chromosome replication are achieved in *Caulobacter*.

FIGURES AND TABLES

Table 2: Cdl-1 and Cdl-2 homologues in other bacterial species.

Cdl-1 Homologues	Cdl-2 Homologues
Escherichia coli *Salmonella typhimurium* *Shigella flexneri* *Yersinia pestis* *Erwinia carotovora* *Azotobacter vinelandii* *Haemophilus influenzae* *Pseudomonas aeruginosa* *Actinobacillus pleuropneumoniae* *Legionella pneumophila* *Agrobacterium tumefaciens* *Brucella abortus* *Mesorhizobium loti* *Maricaulis maris* *Xanthobacter autotrophicus* *Sinorhizobium meliloti* *Rhodobacterales sphaeroides*	*Agrobacterium tumefaciens* *Brucella abortus* *Mesorhizobium loti* *Maricaulis maris* *Xanthobacter autotrophicus*

Figure 1. *Homology of DnaA-like genes. E. coli* DnaA is comprised of four domains. The oligomerization, DnaB binding, ATPase, and DNA binding domains are shown (21). The homology of *E. coli* and *C. crescentus* with DnaA is illustrated. Also shown is the homology between Cdl-1 (Hda: CC1711) and Cdl2 (CC1058) with the *Caulobacter* DnaA and shows Cdl-1 is homologous to the ATPase domain of *Caulobacter* DnaA and Cdl-2 shares homology with the DNA binding domain of *Caulobacter* DnaA.

DnaA-like Genes in *Caulobacter crescentus*

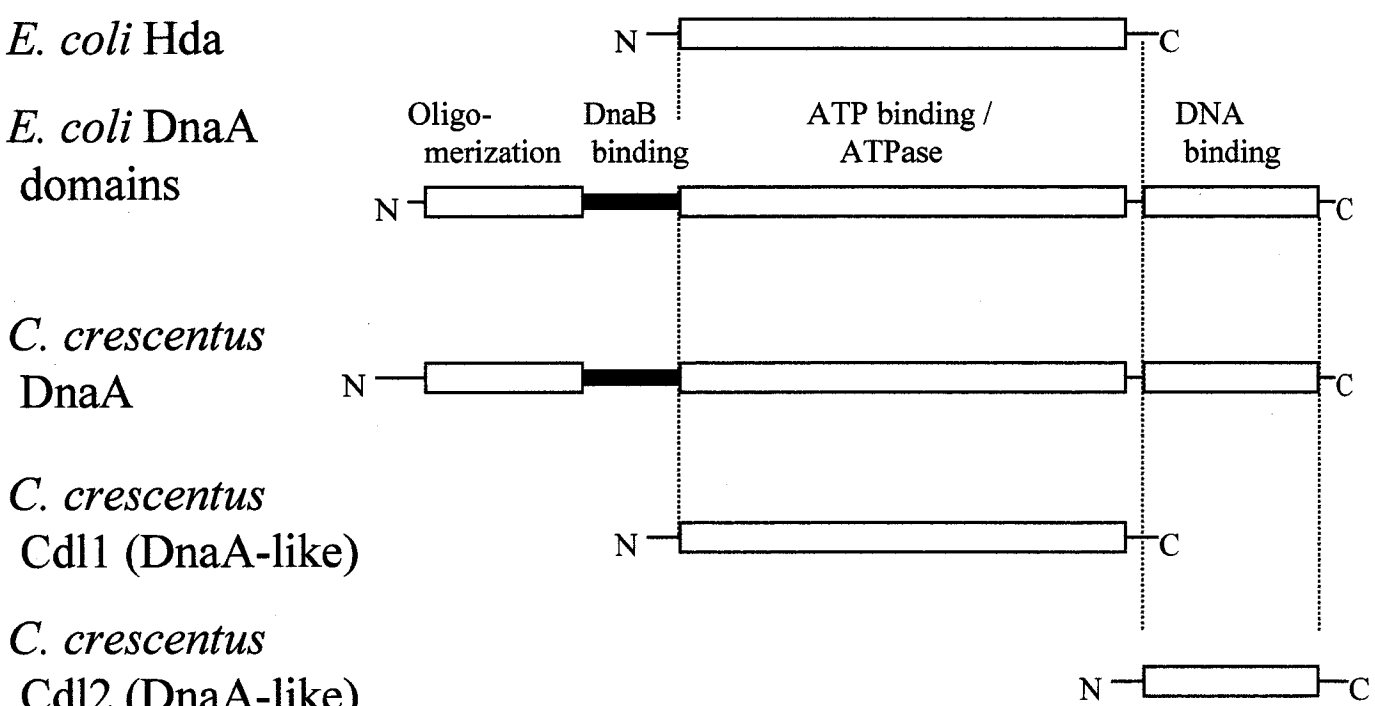


Figure 2. *Cdl-1 (Hda) and Cdl-2 homologues from other bacteria.* A) Sequence alignments of Cdl-1 protein homologues from other bacteria.. The alignments reveal two major sub-domains. The first comprises the Walker A (A) and Walker B (B) motifs that are involved in nucleotide binding and hydrolysis respectively (22). The second sub domain includes Sensor I (I), Sensor II (II), and Box VII (VII) that represent conserved sequence elements found among AAA+ family members (23). B) Alignment of Cdl-2 homologues. Cdl-2 is restricted to the alpha-proteobacteria group, sharing strong homology with the Domain IV DNA-binding domain (DBD) of DnaA (21). The scale on the bottom of each panel indicates the number of amino acids.

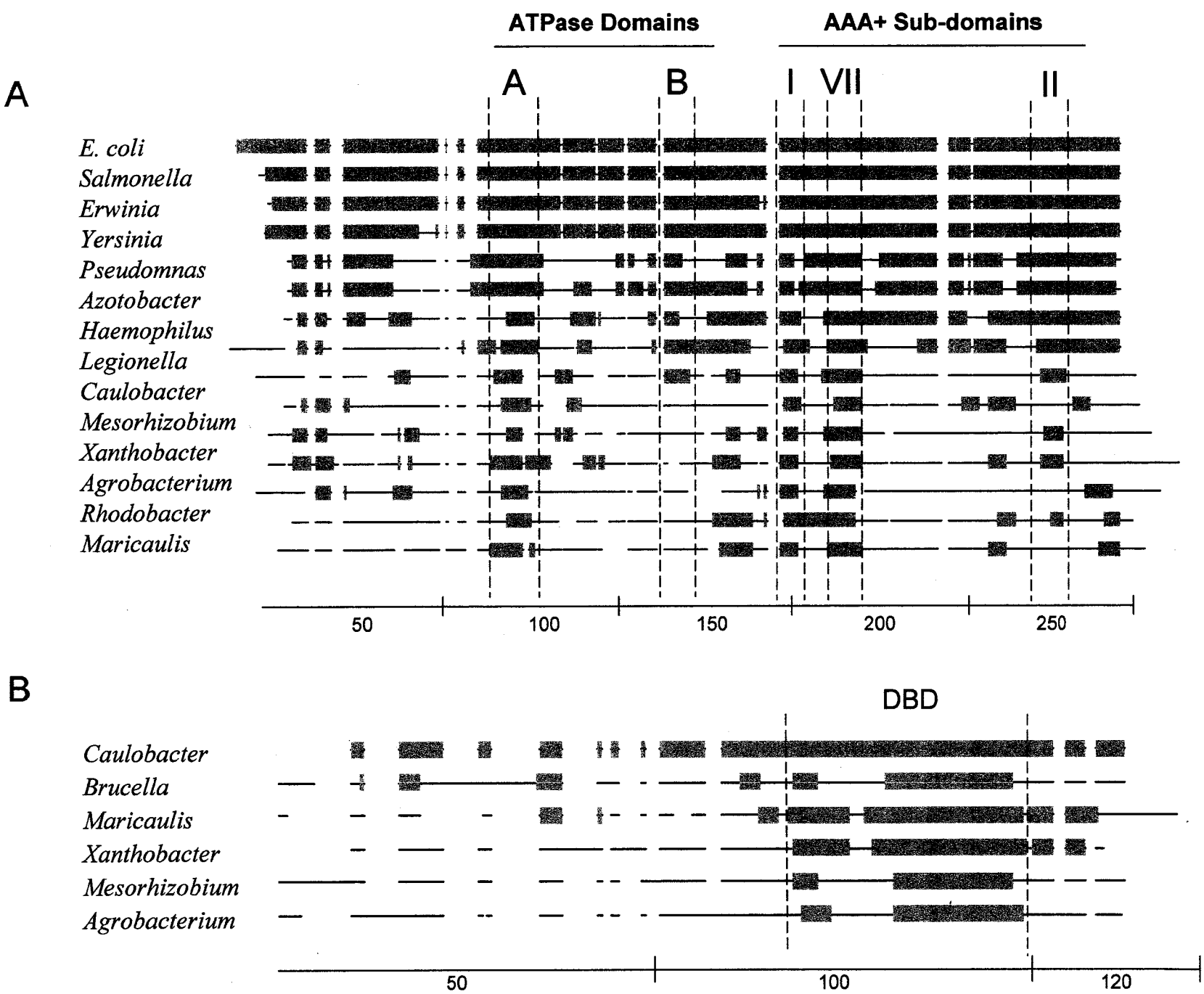
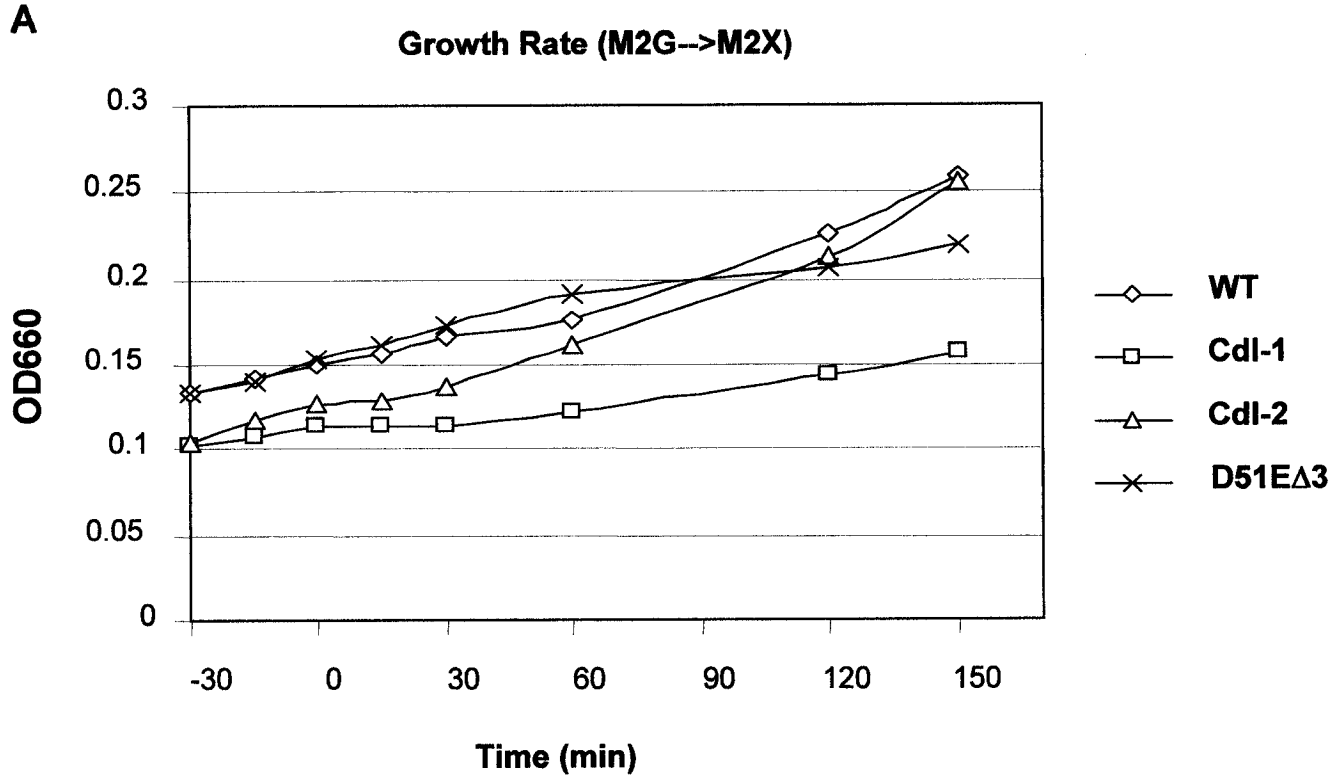

A
ATPase Domains
AAA+ Sub-domains
A
B
I
VII
II
E. coli
Salmonella
Erwinia
Yersinia
Pseudomnas
Azotobacter
Haemophilus
Legionella
Caulobacter
Mesorhizobium
Xanthobacter
Agrobacterium
Rhodobacter
Maricaulis
50
100
150
200
250
B
DBD
Caulobacter
Brucella
Maricaulis
Xanthobacter
Mesorhizobium
Agrobacterium
50
100
120

Figure 3. *Over-expression of DnaA-like genes Cdl-1 and Cdl-2 on growth rate and DNA synthesis in Caulobacter.* Cells were grown exponentially at 30°C in minimal media in glucose (0.1%) and compares the growth and DNA synthesis rates of WT, Cdl1, Cdl2, and CtrA D51EΔ3-expressing cells before and after induction with 0.1% xylose. A) Cell growth at A_{660}. B) DNA synthesis, plotted as incorporation of P^{32} alpha-dCTP/A_{660} vs. time in culture.

A


Growth Rate (M2G-->M2X)
OD660
0.3
0.25
0.2
0.15
0.1
0.05
0
-30
0
30
60
90
120
150
Time (min)
WT
Cdl-1
Cdl-2
D51EΔ3

B

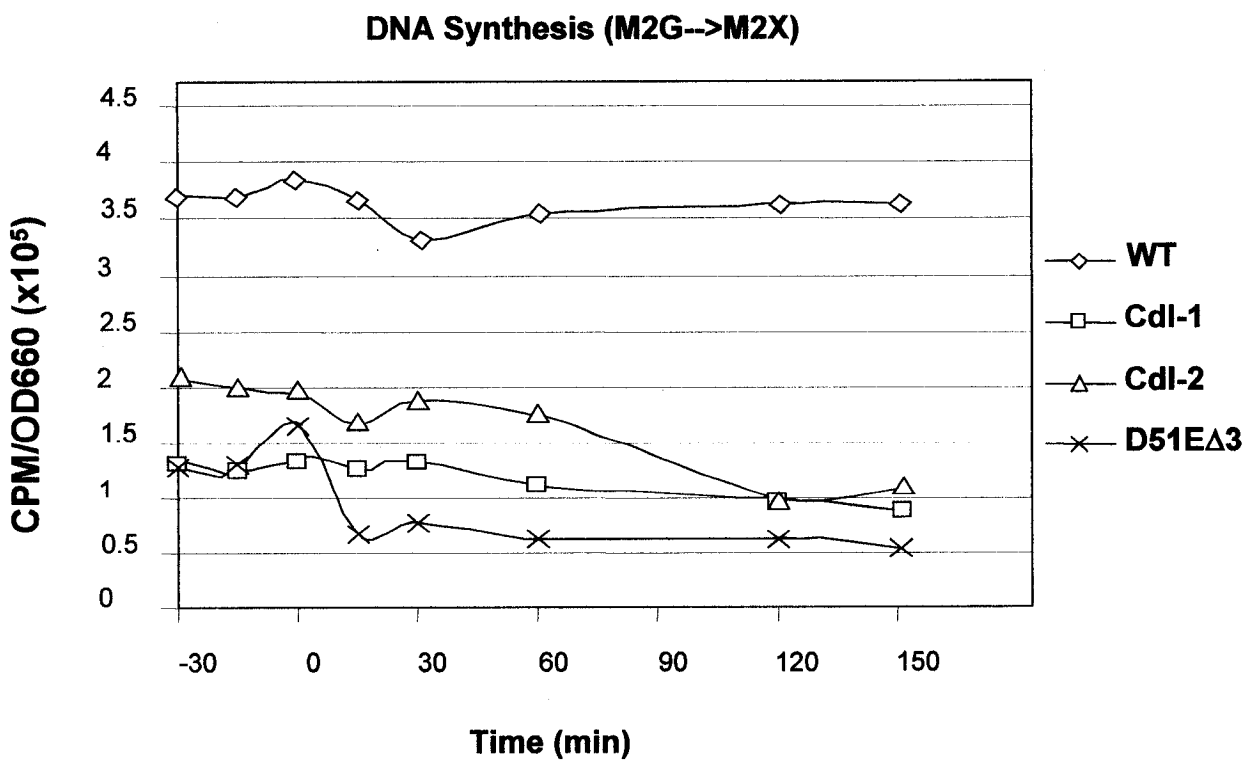
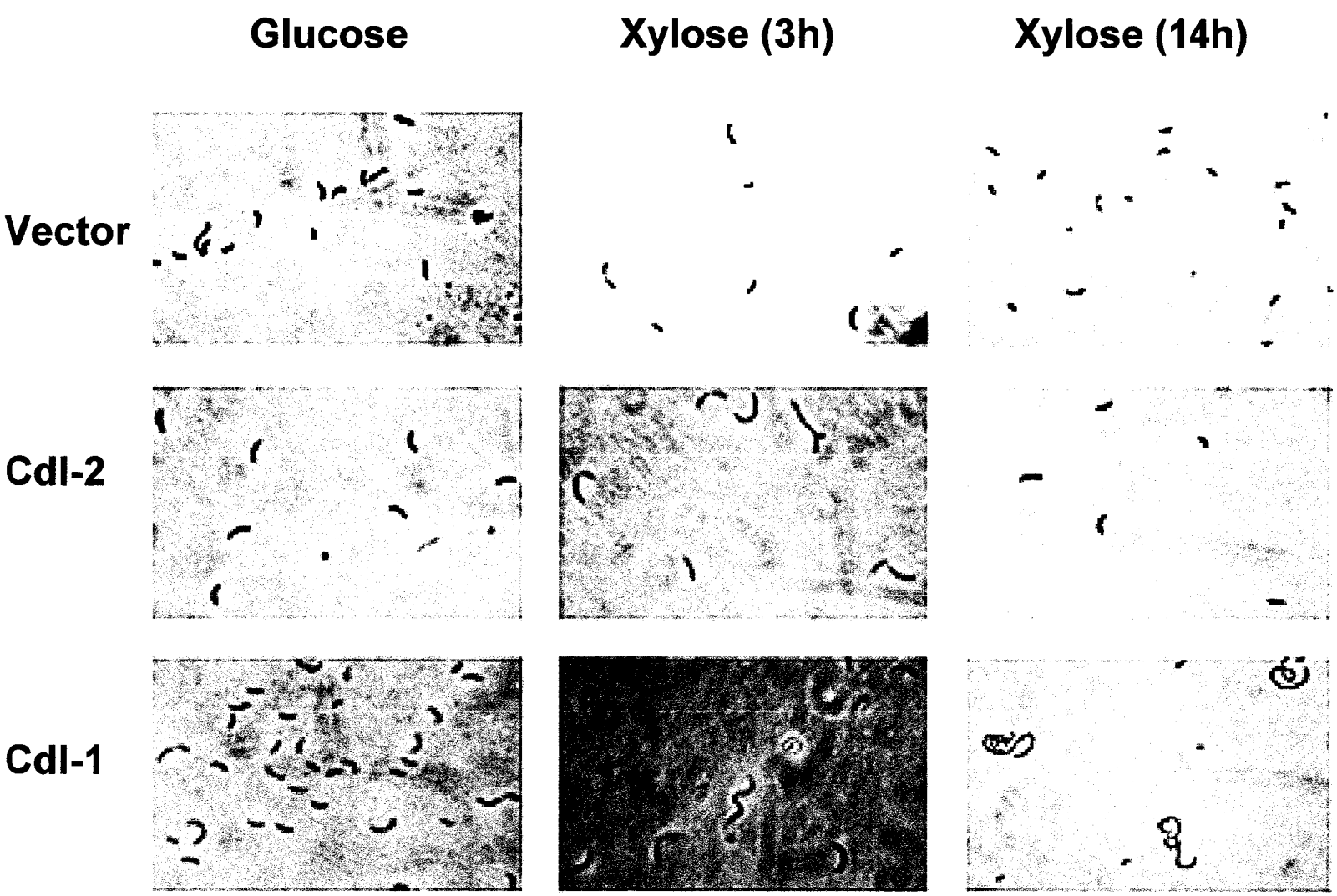

DNA Synthesis (M2G-->M2X)
CPM/OD660 (x10⁵)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-30
0
30
60
90
120
150
Time (min)
WT
Cdl-1
Cdl-2
D51EΔ3

Figure 4. *Overexpression of Cdl-1 and Cdl-2 induce growth defects in Caulobacter.* Exponentially growing WT, Cdl-1 and Cdl-2-expressing cells were transferred to minimal media with 0.1% xylose and grown at 30°C. Cells were harvested at 3 and 14 hours post induction and digitally photographed under phase contrast (magnification: 100X oil immersion).


Glucose
Xylose (3h)
Xylose (14h)
Vector
Cdl-2
Cdl-1

REFERENCES

1. Messer, W. and Weigel, C. (1996) In Neidhardt, F. C., Curtiss III, R., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M. and Umbarger, H. E. (eds.), *Escherichia coli and Salmonella, Cellular and Molecular Biology*. ASM Press, Washington, D.C., Vol. 1, pp. 1579-1601.

2. Slater, S., Wold, S., Lu, M., Boye, E., Skarstad, K. and Kleckner, N. (1995) *E. coli* SeqA protein Binds *oriC* in Two Different Methyl-Modulated Reactions Appropriate to Its Roles in DNA Replication Initiation and Origin Sequestration. *Cell*, **82**, 927-936.

3. Boye, E., Lobner-Olesen, A. and Skarstad, K. (2000) Limiting DNA replication to once and only once. *EMBO Reports*, **1**, 479-483.

4. Kitagawa, R., Ozaki, T., Moriya, S. and Ogawa, T. (1998) Negative control of replication initiation by a novel chromosomal locus exhibiting exceptional affinity for *Escherichia coli* DnaA protein. *Genes and Development*, **12**, 3032-3043.

5. Messer, W. (2002) The bacterial replication initiator DnaA. DnaA and oriC, the bacterial mode to initiate DNA replication. *FEMS Microbiol Rev*, **26**, 355-374.

6. Camara, J.E., Skarstad, K. and Crooke, E. (2003) Controlled Initiation of Chromosomal Replication in Escherichia coli Requires Functional Hda Protein. *J Bacteriol*, **185**, 3244-3248.

7. Kato, J. and Katayama, T. (2001) Hda, a novel DnaA-related protein, regulates the replication cycle in *Escherichia coli*. *The EMBO Journal*, **20**, 4253-4262.

8. Katayama, T., Kubota, T., Kurokawa, K., Crooke, E. and Sekimizu, K. (1998) The Initiator Function of DnaA Protein Is Negatively Regulated by the Sliding Clamp of the *E. coli* Chromosome Replicase. *Cell*, **94**, 61-71.

9. Camara, J.E., Breier, A.M., Brendler, T., Austin, S., Cozzarelli, N.R. and Crooke, E. (2005) Hda inactivation of DnaA is the predominant mechanism preventing hyperinitiation of *Escherichia coli* DNA replication. *EMBO Reports*, **6**, 736-741.

10. Gorbatyuk, B. and Marczynski, G.T. (2001) Physiological consequences of blocked *Caulobacter crescentus* DnaA expression, an essential DNA replication gene. *Molecular Microbiology*, **40**, 485-497.

11. Meisenzahl, A.C., Shapiro, L. and Jenal, U. (1997) Isolation and Characterization of a Xylose-Dependent Promoter from *Caulobacter crescentus*. *Journal of Bacteriology*, **179**, 592-600.

12. Simon, R., Priefer, U. and Puler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: Transposon mutagenesis in Gram negative bacteria. *Biotechnology*, **1**, 784-791.

13. Guenther, B., Onrust, R., Sali, A., O'Donnell, M. and Kuriyan, J. (1997) Crystal Structure of the [delta]' Subunit of the Clamp-Loader Complex of E. coli DNA Polymerase III. *Cell*, **91**, 335-345.

14. Erzberger, J.P. and Berger, J.M. (2006) EVOLUTIONARY RELATIONSHIPS AND STRUCTURAL MECHANISMS OF AAA+ PROTEINS. *Annual Review of Biophysics and Biomolecular Structure*, **35**, 93-114.

15. Su'etsugu, M., Shimuta, T.R., Ishida, T., Kawakami, H. and Katayama, T. (2004) Protein associations in DnaA-ATP hydrolysis mediated by the replicase clamp-Hda complex. *J Biol Chem.*

16. Domian, I.J., Quon, K.C. and Shapiro, L. (1997) Cell type-specific phosphorylation and proteolysis of a transcriptional regulator controls the G1 to S transition in a bacterial cell cycle. *Cell*, **90**, 415-424.

17. Marczynski, G.T. and Shapiro, L. (2002) Control of chromosome replication in *Caulobacter crescentus*. *Annual Reviews in Microbiology*, **56**, 625-656.

18. Quon, K.C., Yang, B., Domian, I.J., Shapiro, L. and Marczynski, G.T. (1998) Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin. *Proc. Natl. Acad. Sci. USA*, **95**, 120-125.

19. Messer, W., Blaesing, F., Jakimowicz, D., Krause, M., Majka, J., Nardmann, J., Schaper, S., Seitz, H., Speck, C., Weigel, C. *et al.* (2001) Bacterial replication initiator DnaA. Rules for DnaA binding and roles of DnaA in origin unwinding and helicase loading. *Biochimie*, **83**, 5-12.

20. Gorbatyuk, B. and Marczynski, G.T. (2005) Regulated degradation of chromosome replication proteins DnaA and CtrA in Caulobacter crescentus. *Mol Microbiol*, **55**, 1233-1245.

21. Messer, W., Blaesing, F., Majka, J., Nardmann, J., Schaper, S., Schmidt, A., Seitz, H., Speck, C., Tungler, D., Wegrzyn, G. *et al.* (1999) Functional domains of DnaA proteins. *Biochimie*, **81**, 819-825.

22. Walker, J., Saraste, M., Runswick, M.J. and Gay, N.J. (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase,

myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J*, **1**, 945-951.

23. Neuwald, A.F., Aravind, L., Spouge, J.L. and Koonin, E.V. (1999) AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Research*, **9**, 27-43.

CHAPTER V: THESIS SUMMARY

The introduction to this thesis promotes the utility of *Caulobacter crescentus* in our understanding and investigation of DNA replication because it provides an accessible genetic system for the study of how chromosome replication is coupled to an organism's cell growth and development. The homologous features of DNA replication among all living organisms studied, allows us to draw important parallels between *Caulobacter* replication and that of higher organisms. We understand that unregulated DNA replication leads to loss of viability both at the cellular level and at the organismal level. For instance, in humans, defects in the replication cycle can lead to serious and often debilitating neurological disorders (1) and cancer (2).

Thematically, this thesis draws similarities and differences between prokaryotic and eukaryotic DNA replication but also between regulatory mechanisms used in different bacterial systems. In *E. coli,* similar to *Caulobacter*, chromosome replication is controlled by the DnaA protein. However, in *E. coli,* initiation of chromosome replication can occur more than once per cell cycle. Functional homology between *E. coli* and *Caulobacter* suggests nature has conserved the framework for DNA replication but the regulatory mechanisms controlling this process are as varied as the organisms themselves. Why this difference exists between *Caulobacter* and *E. coli* perhaps derives from the environmental conditions that each organism lives in. *Caulobacter* is conditioned to growth in dilute aquatic environments where a single cell cycle may take as long as eight hours (compared to two hours in the warm comfort of our laboratory) and are reminiscent of cells in stationary phase. Under the microscope, these 'wild' *Caulobacter* populations are primarily found as non-replicating stalk cells, surprisingly,

the same cell type that is competent for replication. We might argue, based on studies done with stationary phase cells (data not shown), that in these quiescent cells, CtrA is abundant and bound to the origin of replication. Consequently, the DnaA protein (initiator) is not present in these cells due to regulated proteolysis (3). Therefore, under conditions of limited nutrient availability, the presence of CtrA and the absence of DnaA **reinforces** the restriction of chromosome replication (life is tough model). On the other hand, *E. coli* cycles between aquatic/terrestrial environments and the GI tracts of numerous organisms. Under these conditions nutrient availability in the *E. coli* micro-environment changes dramatically (life is a rollercoaster model). In *E. coli* all regulatory inputs elucidated control the activity of DnaA (4). All three of these mechanisms are not stable and the influence of any of these signals on DNA replication rapidly decays. For instance, sequestration only lasts approximately eight minutes in growing cells (5) presumably because remethylation and the action of chromosome segregation disrupt this activity. Titration is a property of the chromosome (6) and is itself titrated when the two chromosomes are compartmentalized following cell division. The final mechanism, RIDA appears to function only at the initiation to elongation step by converting DnaA-ATP to the inactivated DnaA-ADP form. Unlike *Caulobacter*, *E. coli* does not proteolyze DnaA or express a CtrA homolog. Therefore, the mechanism of reinforcement is not available. As well, the transient nature of these regulatory mechanisms in *E. coli* suggests initiation of chromosome replication is not suppressed per se but only delayed. Such a mechanism makes sense for an organism that must establish rapid cell growth in highly competitive environments. From a global perspective *Caulobacter* uses a tightly regulated mechanism (CtrA turnover and DnaA

replenishment) in response to the slow nutrient fluctuations found in its native environment. However, in *E. coli* the nutrient concentrations can rapidly fluctuate and the selective advantage is given to those organisms that can grow rapidly and divide. For *E. coli* this means an ability to initiate chromosome replication more than once per cell cycle.

The recent Saragossa Sea genome sequencing project reveals that *Caulobacter* and its living descendents may represent the most abundant genetic group on Earth. This implies that *Caulobacter*-like regulation of chromosome replication is a preferred mechanism. Interestingly, in higher eukaryotes, the period between subsequent replication initiation events can extend from days to months. Therefore, our understanding DNA replication in *Caulobacter* opens a door to understanding chromosome replication in higher eukaryotic whose regulatory framework might descend from this primordial and important experimental organism.

This thesis demonstrates for the first time that CtrA phosphorylation does not stimulate binding to a new class of promoters Prior transcription models have suggested that phosphorylation increases the affinity of response regulators for target DNA sequences. This model further suggests that promoter occupancy, stabilized by phosphorylation, is the minimal signal required for mRNA transcription. This work shows that constitutive *in vivo* occupancy of the *ctrA* promoters by the non-proteolyzable mutant, CtrAΔ3, did not alter the cell cycle pattern of *ctrA* gene transcription. These results support a revised model of CtrA-regulated transcription and suggest an allosteric model of transcriptional

is utilized. Allosteric regulation implies a role of protein-protein contacts and future work should define the functional unit of CtrA and whether CtrA can bind DNA as a dimer, tetramer, or higher order oligomer. The *ctrA* promoters are not unique among CtrA-regulated genes having unusual CtrA binding sites and further analysis of other promoters such as the flagellar genes *motB* and *fliX* (only a single TTAA half site) may reveal new members to this class of promoters.

Chapter 3 provides convincing evidence that CtrA interacts with *Cori in vivo* and that CtrA does not occupy *Cori* in stalk cells when chromosome replication commences. This chapter also demonstrates, for the first time, that a chaperone (ClpX) can be recruited to a chromosome replication origin. Future work should confirm the role of ClpX in the release of CtrA from *Cori*. Dnase I footprints of *Cori* using purified CtrA and ClpX may address how ClpX modulates CtrA binding to *Cori*. CtrA-mediated recruitment of ClpX suggest ClpX may contact other proteins in the replication origin, such as DnaA. Further work along these lines may reveal the steps necessary to de-repress chromosome replication in stalk cells.

The identification of *cdl-1* and *cdl-2* proposes that a RIDA-like mechanism functions in *Caulobacter*. The most important experiments for this project are in-frame deletions of both *cdl-1* and *cdl-2* to ascertain whether these genes are essential in *Caulobacter*. Conditional strains should also be constructed which allow for selective down regulation of these genes individually or together to assess the impact on chromosome replication.. The DNA binding domain homologue, Cdl-2, should also be investigated for possible

DNA binding activity. A GST-Cdl-2 fusion protein has recently been tested in Dnase I footprints of *Cori* but did not demonstrate binding. These experiments should be repeated with purified Cdl-2 without the GST fusion protein. It is also possible to address DnaA-ATP/ADP exchange and it would be interesting to determine whether Cdl-1, Cdl-2, or both influence DnaA ATPase activity *in vitro*.

Taken together, the data suggest the following simple model for *Caulobacter* replication. CtrA utilizes allosteric properties to control gene transcription and suggests these properties may be utilized to communicate with proteins, such as ClpX, at the replication origin. During the swarmer to stalk transition, ClpX enters the replication origin and mediates the release of CtrA from *Cori*. Immediately following the commencement of chromosome replication, CtrA is not available to bind *Cori* and a second mechanism, RIDA, may provide the necessary signals to deactivate DnaA and prevent re-initiation. I hope that future research in our lab continues to work towards elucidating the steps necessary for the derepression of chromosome replication in stalk cells as well as to gain a better understanding of how chromosome replication is limited to once and only once achieved in *Caulobacter* and other organisms.

References

1. Krasilnikova, M.M. and Mirkin, S.M. (2004) Replication Stalling at Friedreich's Ataxia (GAA)n Repeats In Vivo. *Mol. Cell. Biol.*, **24**, 2286-2295.

2. DePamphilis, M.L.e. (2006) *DNA Replication and Human Disease*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

3. Gorbatyuk, B. and Marczynski, G.T. (2005) Regulated degradation of chromosome replication proteins DnaA and CtrA in Caulobacter crescentus. *Mol Microbiol*, **55**, 1233-1245.

4. Messer, W., Blaesing, F., Jakimowicz, D., Krause, M., Majka, J., Nardmann, J., Schaper, S., Seitz, H., Speck, C., Weigel, C. *et al.* (2001) Bacterial replication initiator DnaA. Rules for DnaA binding and roles of DnaA in origin unwinding and helicase loading. *Biochimie*, **83**, 5-12.

5. Skarstad, K., Lueder, G., Lurz, R., Speck, C. and Messer, W. (2000) The *Escherichia coli* SeqA protein binds specifically and co-operatively to two sites in hemimethylated and fully methylated *oriC*. *Molecular Microbiology*, **36**, 1319-1326.

6. Ogawa, T., Yamada, Y., Kuroda, T., Kishi, T. and Moriya, S. (2002) The *datA* locus predominantly contrbutes to the initiator titration mechanism in the control of replication initiation in *Escherichia coli*. *Mol Microbiol*, **44**, 1367-1375.

APPENDIX A: RESEARCH COMPLIANCE CERTIFICATES

1. APPLICATION TO USE BIOHAZARDOUS MATERIALS
2. INTERNAL RADIOISOTOPE PERMIT